ASSESSING THE POTENTIAL OF *SALIX ALAXENSIS* FOR THE RHIZOREMEDIATION OF DIESEL-CONTAMINATED SOIL

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A

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Abstract

Alaska has over 280 remote communities that rely on diesel as their main source of heat; as a result, there have been multiple diesel spills across the state. Research has shown that plants are able to assist in the degradation of diesel through rhizoremediation, relying on the interaction between microorganisms, plant roots, and other components of the soil environment. Greater attention is now being given to the potential role of secondary plant compounds released during fine root turnover and the stimulatory effects they may have on the rhizoremediation process. For this study the native plant species, *Salix alaxensis* (felt leaf willow) was chosen. Fine root turnover in the sub-Arctic was mimicked through a microcosm study performed with sub-Arctic soil contaminated with weathered and fresh diesel, incubated at 4 °C and 20 °C. The effect of adding crushed willow roots was compared against addition of pure salicylic acid, a secondary plant compound found in the salicaceae family, and/or addition of fertilizer. Results showed that the addition of crushed fine willow roots with or without fertilizer increased diesel loss. Overall, greater loss and higher respiration occurred at 20 °C. The addition of salicylic acid with or without fertilizer increased soil toxicity. Toxicity may have been the result of observed phenol production and/or fungal growth. Findings show promise for the use of *Salix alaxensis* for the rhizoremediation of diesel contaminated soils in the sub-Arctic.

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Dedication

This thesis is dedicated to my parents, Jeffrey and Cindy Bay, who have been my main support system even when we have been separated by over 3,000 miles. It was through them believing in me that I even attempted obtaining my master's degree. Lastly, I want to dedicate this thesis to Marty who passed away before its completion. Marty was with me from the very beginning, he was there for the long nights in the lab and for the many rough drafts of my thesis, I could not have done this without him.

Section 1 Introduction

1.1 Overview

Diesel is a ubiquitous energy source in rural Alaska; it is used as a fuel for vehicles and heavy equipment, and serves as the primary heating source for most homes and public buildings. Thus, it is not surprising that minor and/or major diesel spills occur frequently throughout Alaska. Each year the Alaska Department of Environmental Conservation (ADEC) receives over 2,000 spill reports for oil and hazardous substances according to the ADEC Prevention, Preparedness and Response Program (PPRP) website (http://www.state.ak.us/local/akpages/ENV.CONSERV./home.htm). However, the ADEC-PPRP notes that this number does not include the spills that go unreported. According to the ADEC SPILLS database (http://www.state.ak.us/local/akpages/ENV.CONSERV./home.htm) only three spills are reported for the community of Kaltag, Alaska, whereas Kaltag residents indicate that many more spills have taken place within the community (Guritz, 2011).

Once released, diesel can have various toxic effects on the environment, wildlife, and humans. Where diesel is spilled, plant growth may be inhibited, there can be detrimental health impacts to land based and aquatic wildlife, and human health may be affected. Acute symptoms of diesel exposure can include, but are not limited to, nausea, eye and skin irritation, and chemical pneumonitis (Hess, 2012). Diesel is an Occupational Safety and Health Administration (OSHA) select carcinogen that targets the kidneys, according to the United States Department of Labor website (https://www.osha.gov/dts/chemicalsampling/data/CH_234655.html). Multiple studies have indicated that components of diesel, specifically the polycyclic aromatic hydrocarbons (PAHs), bioaccumulate in marine organisms (ATSDR, 1995). Further research is required into the ability of PAHs to accumulate in terrestrial organisms (ATSDR, 1995). The

Alaska Health and Social Services Division of Public Health has set allowed exposure levels for common PAHs found in diesel, indicating that chronic exposure to PAHs could negatively impact human health (ADEC, 2012). It is because of detrimental health effects and the potential for diesel compounds to accumulate in organisms that remediation of diesel spills must occur in a timely fashion.

In a location where infrastructure and necessary equipment are present, response to a diesel spill can be quick and remediation of the site can occur faster. However, within Alaska's remote communities, such a fast response is not always feasible due to multiple limiting factors. In most communities, equipment and supplies must either be flown or barged in, and occasionally trained personnel may also be required. This can be expensive in and of itself, and there are additional costs associated with *ex-situ* remediation of the soil.

Common *ex-situ* remediation and disposal options of diesel contaminated soil in Alaska are thermal desorption, landfarming, landspreading and use as landfill cover. Thermal desorption of contaminated soil requires heating excavated soil, at a permitted facility, to an appropriate temperature that allows the hydrocarbons to volatilize. In the state of Alaska, there are currently only two permitted thermal treatment facilities. Landfarming requires excavating contaminated soils, placing the soil in a bermed location with or without a liner, and followed by tilling and fertilizing until remediation is complete (ADEC, 2009). Landspreading requires excavation of contaminated soil and tilling the contaminated soil into the surface layer of a suitable area and allowing the soil to biologically remediate (ADEC, 2009). Another option is to use contaminated soils as landfill cover. When a landfill cell is being closed, excavated contaminated soils can be placed above the waste; the cover soil would be seeded with grasses and over time will be remediated. In order for contaminated soils to be used as landfill cover

specific contaminant concentrations must be met; this can be a limiting factor. Treating soil either through landfarming or thermal desorption has been estimated to cost between $200 – 1,500 per ton of soil in the continental U.S. (Gerhardt et al., 2008), and costs are considerably higher in remote Alaskan communities. The higher costs in remote regions of Alaska result from lack of economic competition, transportation costs and other structural limitations (Goldsmith, 2008).

Because of the high costs associated with typical *ex-situ* methods and the limitations encountered in rural locations, bioremediation is an attractive option. Bioremediation relies on the use of microbes to completely break down the diesel components to carbon dioxide (CO_2) and water (H_2O). In addition to low cost and the potential to achieve complete mineralization, *in-situ* bioremediation allows for the cleanup of a site without disturbing the natural ecology or at least with less ecological effects (Wenzel, 2009).

Phytoremediation, the use of plants in bioremediation, can be employed to assist/enhance bioremediation through a variety of mechanisms further discussed in Section 1.6, including phytostabilization, phytoextraction, phytovolatilization/rhizovolatilization, and rhizoremediation (Wenzel, 2009). Rhizoremediation, the degradation of contaminants within the rhizosphere, is considered to be the dominant process in organic contaminant breakdown when utilizing plants. Degradation that occurs within the rhizosphere is the result of complex interactions between microorganisms, the soil within the rhizosphere, roots, and root exudates (Singer, 2003). There are many benefits to using rhizoremediation, including low treatment cost of $10-50 per ton of contaminated soil (prices based on costs in the continental U.S.), lower maintenance cost, relatively easy implementation (can be used anywhere that plants grow), improved soil quality, and good acceptance by the general public (Gerhardt et al., 2008). Laboratory and field studies

have shown that plants can enhance the microbial biodegradation of petroleum products, (Kuiper et al., 2004; Arthur et al., 2005).

Root exudates, consisting of sugars, organic acids and other organic compounds, are commonly released into the rhizosphere through the roots and are used by microbes as carbon and energy sources. Root exudates and associated compounds may also be released into the rhizosphere through fine root turnover. Many of these compounds are aromatics that are similar in structure to organic contaminants and can even be identical to intermediate products produced during microbial breakdown of the contaminants (Singer et al., 2004). Salicylic acid is a common compound released by willows and, in addition, is an intermediate product of naphthalene breakdown and inducer of naphthalene degradation gene expression (Chen and Aitken, 1999).

1.2 Study Hypothesis and Objectives

The objective of this study is to assess the potential of rhizoremediation of diesel-contaminated soil from sub-Arctic Alaska using a native plant species, *Salix alaxensis* (felt leaf willow), and indigenous soil microorganisms. We hypothesize that fertilization, increased temperature as well as the simulated fine root turnover (i.e. addition of salicylic acid or crushed willow roots) will increase the rate of microbial bioremediation of weathered diesel-contaminated soil. Increased diesel degradation should be indicated through reduced concentration in the soil, increased respiration, and reduced toxicity of the soil. The overarching objective of this study is to determine, based on microcosm studies, whether *S. alaxensis* should be recommended for a phytoremediation field trial for diesel contaminated soil in the community of Kaltag, Alaska. To decide this, the following questions must be answered:

- Are diesel degrading microorganisms (DDM) present in Kaltag, AK soil and can those present assist in rhizoremediation, even at environmentally relevant temperatures?
- What is the intrinsic diesel degradation of the weathered-diesel and fresh-diesel contaminated soil from Kaltag, AK?
- Is the weathered-diesel contaminated soil from Kaltag, AK toxic to microorganisms? Will intrinsic biodegradation or simulated rhizoremediation reduce the toxicity of diesel-contaminated soil?
- Does the addition of crushed fine willow roots, salicylic acid and/or fertilizer increase diesel loss and CO_2 production?

1.3 Aerobic Diesel Degradation Metabolic Pathways

Diesel is refined from crude oil through distillation processes and consists strictly of hydrocarbons, specifically $C_{10} - C_{25}$ (ADEC, 2002) that have a boiling point range of 170 °C to 400 °C. The dominant hydrocarbons in diesel are aliphatics (~64%), aromatics (~35%) and a small amount of olefinic hydrocarbons (1-2%) (ATSDR, 1995). As diesel is composed of hydrocarbons, it is mostly non-polar and stable at room temperature (Sierra-Garcia and de Oliveira, 2013). Once released into the environment, diesel can be degraded by both abiotic and biotic processes (Wang et al., 2003).

A wide variety of microbes, both prokaryotic and eukaryotic, are capable of biodegrading petroleum hydrocarbons. They can rely on aerobic or anaerobic processes, with aerobic biodegradation being the most rapid and significant process in most soil environments. Prokaryotes have been found to degrade diesel include species belonging to the phyla *Actinobacteria*, *Firmicutes, Proteobacteria, Acidobacteria,* and *Chloroflexi* (Sutton et al., 2013,

Prince et al., 2010). Archaea, specifically those belonging to the phylum *Eukarchaeota*, were found by Sutton et al. (2013) to degrade diesel. *Cyanobacteria* have been found to degrade diesel on a small scale, however it is not known at this time if the *Cyanobacteria* play a significant role in environmental biodegradation processes (Prince et al., 2010). Eukaryotic organisms capable of degrading diesel tend to be fungal, including the phyla *Ascomycota* and *Basidiomycota* (Prince et al., 2010), as well as those belonging to the genera *Candida, Rhodotorula, Aueroebasidium, Sporobolomyces, Penicillium, Aspergillus,* and *Cladosporium* (Atlas and Cerniglia, 1995).

Both alkane and aromatic biodegradation processes are initiated through a mono- or di-oxygenase reaction, which relies on oxygen as a co-substrate (Sierra-Garcia and de Oliveira, 2013). Examples of alkane and aromatic pathways are shown in Figure 1 and Figure 2. The key enzyme in the alkane degradation pathway is the alkane monooxygenase that terminally oxidizes the alkane to form a 1-alkanol (Whyte et al., 2002a). There are several types of monooxygenase enzymes, but the best characterized is the AlkB monoxygenase (*alkB*), typically found to degrade medium length hydrocarbon chains (C3-C10 or C10-C20) (Sierra-Garcia and de Oliveira, 2013). Many of the monooxygenase genes detected in petroleum-contaminated soils are related to the *alkB* genes, there is a significant difference in the gene relations between gram-positive and gram-negative organisms (Whyte et al., 2002b, Smits et al., 2002). The monooxygenase functions as part of a three component system, consisting of the integral-membrane alkane hydroxylase (*alkB*/AlkB), rubredoxin (*alkG*/AlkG) and rubredoxin reductase (*alkT*/AlkT) (Whyte et al., 2002a; Smits et al., 2002).

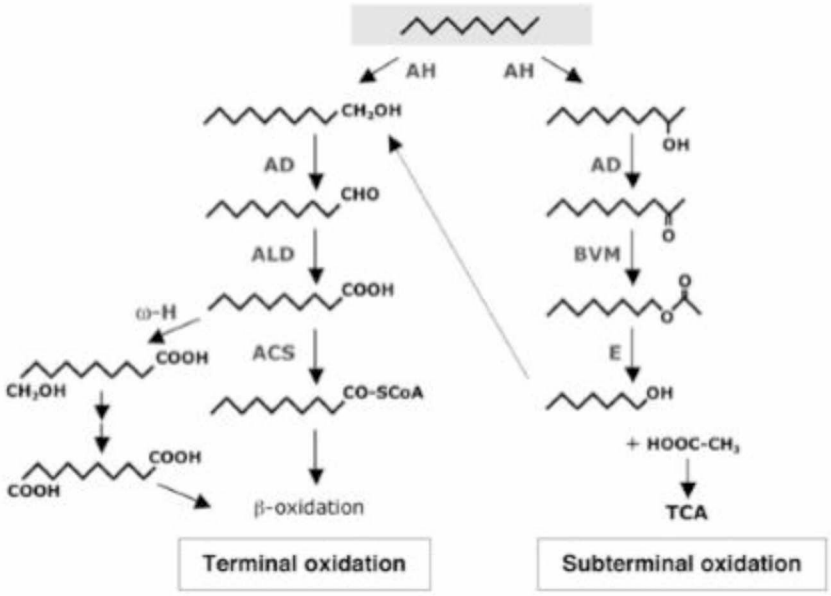

Figure 1: Example of Alkane Degradation Pathway (Rojo, 2009)

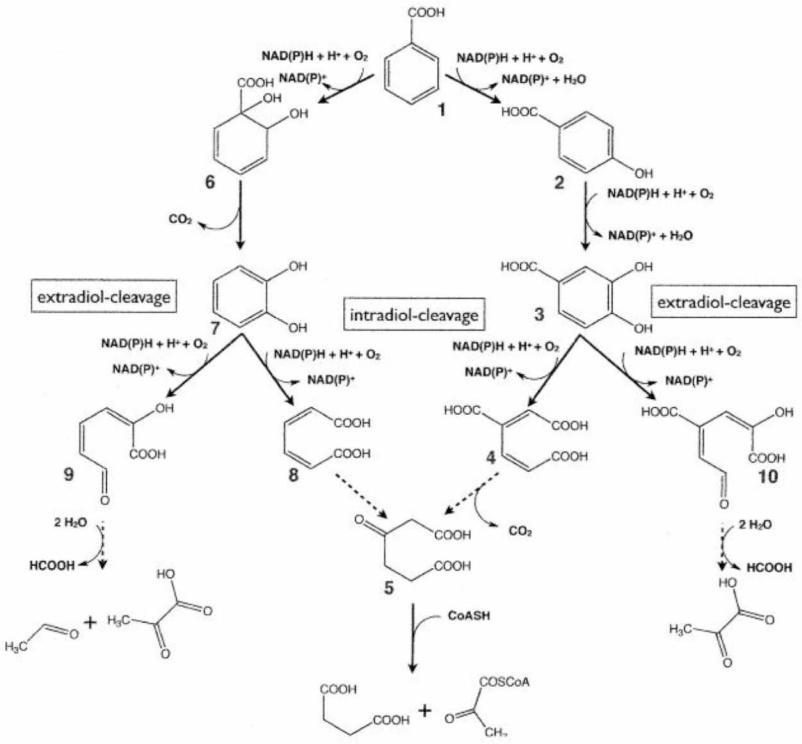

Figure 2: Example of Aromatic Degradation Pathway (Ismail and Gescher, 2012)

Aromatic hydrocarbon breakdown begins with the addition of oxygen to the ring structure by a dioxygenase or an iron (Fe_2) or flavin monooxygenase, causing the hydroxylation of the ring structure (Ní Chadhain et al., 2006; Sierra-Garcia and de Oliveira, 2013). The addition of oxygen forms a *cis*-dihydrodiol that is eventually rearomatized to a dehydroxylated intermediate by a *cis*-diol dehydrogenase (Habe and Omori, 2003). Dioxygenases are multicomponent enzymes, like the monooxygenases, which are made up of an electron transport chain with a ferredoxin, a ferredoxin reductase and a terminal ring hydroxylating dioxygenase (Zhout et al., 2006; Ní Chadhain et al., 2006). Terminal dioxygenases are composed of two subunits, an α- and a β-subunit. The α-subunits are the more conservative catalytic component and contains two regions; the first is the [Fe_2-S_2] Rieske center necessary for the transportation of electrons from the ferredoxin to the second region the mononuclear iron containing domain (Zhou et al., 2006; Ní Chadhain et al., 2006). The α-subunit is also the more critical subunit for substrate recognition (Zhou et al., 2006).

Depending on how the alkanes are activated, they will proceed through two different pathways. An alkane that is terminally oxidized (by an alkane monooxygenase) will next be oxidized to a primary alcohol through substrate specific terminal monooxygenase (Sierra-Garcia and de Oliveira, 2013). Following this step the primary alcohol is then subsequently oxidized into an aldehyde and converted to carboxylic acid (fatty acid), which is then converted to acetyl-CoA (Sierra-Garcia and de Oliveira, 2013). Acetyl-CoA is also produced from the β-oxidation of fatty acids and the final products are then used in the Tricarboxylic Acid cycle (TCA) (Rojo, 2010). Should the alkane be sub-terminally oxidized, it is oxidized to a secondary alcohol, then to a ketone and finally to an ester (Sierra-Garcia and de Oliveira, 2013). The ester can then be oxidized to either a primary alcohol or fatty acid; once this is done, it follows the same steps as

for the terminally oxidized alkane (Rojo, 2010). Some microorganisms are capable of both terminal and sub-terminal oxidation (Sierra-Garcia and de Oliveira, 2013).

The metabolic pathway for aromatic hydrocarbon break down can proceed through different peripheral pathways. After activating the aromatic compound there can be a mono- or dihydroxylation of the ring, the ring is then cleaved either by intradiol dioxygenase or by extradiol dioxygenase (Sierra-Garcia and de Oliveira, 2013). The dioxygenase forms *cis*-diols through the addition of two oxygen molecules to the aromatic structure (Gibson and Parales, 2000). Following the cleaving of the ring structure (meta- or ortho-), the product proceeds through central metabolic pathway reactions and then enter the β-oxidation pathway and then the TCA cycle (Atlas and Cerniglia, 1995). Another option after activating the aromatic structure is to enter the Co-A thioester pathway and the Co-A thioester rings are then further reduced (Sierra-Garcia and de Oliveira, 2013). The final products enter the β-oxidation pathway and the TCA cycle.

1.4 Impacts of Sub-Arctic Conditions on Bioremediation

Interior Alaska is an environment characterized by extreme cold winter temperatures and short, warm summers. Throughout most of Alaska, permafrost can be found as well as periglacial features that result from freeze-thaw cycles (Mohn and Stewart, 2000). Above the permafrost there is an active layer which thaws during the summer and temperatures within this zone have the potential to reach 20 °C (Mohn and Stewart, 2000; Walworth et al., 2001). Spills that occur within the sub-arctic during the summer months tend to be acute and have shorter term impacts, whereas spills during winter conditions are chronic and have long term effects (Yang et al., 2009). Extremely cold temperatures can impact the nature of the diesel. At low temperature

the viscosity of diesel increases, thereby decreasing diesel movement in the soil. Volatilization is also decreased by cold temperatures, which can affect diesel toxicity (Margesin and Schinner, 2001). In addition, cold region soils typically have a low organic content that directly results in decreased sorption of the diesel to the soil (Mohn and Stewart, 2000).

Cold temperature impacts more than the characteristics of diesel, it also impacts the microbial community composition, rate of hydrocarbon degradation, and substrate and electron acceptor mass transfer (Yang et al., 2009). Under extreme cold conditions *in-situ* hydrocarbon degradation has been found to be the result of indigenous cold-adapted psychrophilic and psychrotrophic microbes. Psychrophiles do not grow above 20 °C and optimum growth occurs at ≤ 15 °C, whereas psychrotrophs (psychrotolerant) have optimum growth temperatures between 15 °C and 20 °C. These microorganisms are capable of diesel remediation under both winter and summer conditions (Margesin and Schinner, 2001). The most commonly reported Gram-negative microbes in cold environments include α-, β- and γ-*Proteobacteria* as well as members of the *Cytophaga-Flavobacterium-Bacteriodes* phylum (D'Amico et al., 2006). Commonly found gram-positive microorganism are *Corynebacterium*, *Arthrobacter* sp., and *Micrococcus* sp. (D'Amico et al., 2006). Although these microorganisms may be adapted to cold weather, past studies have shown that the rate of diesel degradation in the sub-arctic is higher during the warmer summer months (15-20 °C) compared the cold winter months (0-5 °C) (Yang et al., 2009; Walworth et al., 2001).

In order for many microorganisms to be able to degrade diesel, they must be able to transfer diesel, or diesel breakdown products generated by extracellular enzymes, across their membrane surface. Extreme cold temperatures can greatly limit the bioavailability of the diesel by impacting the diesel characteristics as well as the physiology of the microbe. When temperatures

reach freezing, the cytoplasm can experience cryogenic stress and cell membrane channels may close, eventually causing a loss of function (Yang et al., 2009; D'Amico et al., 2006). This ultimately prevents the transport of diesel into the cell where intracellular biodegradation occurs. In addition, protein synthesis can be impacted through reduced transcription activity and protein folding (D'Amico et al., 2006), which would affect both intracellular and extracellular biodegradative processes.

Aerobic conditions are favorable for the biodegradation of diesel since they result in the greatest energy yield and highest biodegradation rates. During sub-arctic winter conditions, oxygen diffusion may be limited because of the frozen ground, and the oxygen that is present may be consumed faster than it is replaced (Yang et al., 2009).

1.5 Gene Regulation

Enzymes are crucial to catalysis of metabolic pathways, and their production is controlled by gene regulation. Genes may be expressed continually (constitutively) or subject to regulatory mechanisms that permit expression only under specific circumstances. Studies have shown that in arctic conditions the most abundant microbes containing alkane monooxygenase genes included *Rhodococcus* spp. (present when soils are clean and when contaminated with diesel), *Pseudomonas* spp. (present after diesel contamination) and *Acinetobacter* spp. (present after diesel contamination) (Margesin and Schinner, 2001). The most common alkane biodegradation genes found among these microbes include *alkB, alkM, alkB1 and alkB2* (Margesin et al., 2003). The *alkB* gene is well defined within the *Pseudomonas* spp. and is the first of the *alkBFGHJKL* genes located on the OCT plasmid (Kok et al., 1988; Ratajczak et al., 1998). The alkane hydroxylase terminally oxidizes C_6-C_{12} alkanes, producing a primary alcohol that follows the

previously mentioned metabolic pathways (Kok et al., 1988). The longer chain alkanes (> C_{12}) are terminally oxidized by an alkane hydroxylase encoded by *alkM*, this gene has been well defined in *Acinetobacter* spp. (Margesin et al., 2003; Ratajczak et al., 1998). The *alkM* gene is regulated by the *alkR* gene; both are distinct and are found in a chromosome where they are located opposite of each other (Ratajczak et al., 1998). The alkane hydroxylase system in the *Rhodococcus* spp. relies on monooxygenase gene homologs. The *alkB1* and *alkB2* homologs are part of an *alk* gene cluster; there is an *alkB3* and *alkB4* homolog however they are not a part of the *alk* gene clusters (Whyte et al., 2002a). Each *alkB1* and *alkB2* homolog codes for putative TetR transcriptional regulatory proteins, two rubredoxins, and *alkB1* codes for rubredoxin reductase (Whyte et al., 2002a). *Rhodococcus* spp. are capable of sub-terminally oxidizing alkane chains as well as branched alkanes (C_8 – C_{32}) (Whyte et al., 2002b).

Genes coding for oxygenase enzymes necessary for aromatic degradation are substrate specific (Baldwin et al., 2003). Dioxygenases are made of a ferredoxin electron transport chain, reductase and terminal dioxygenase (Ni Chadhain et al., 2006). Examples of dioxygenases include naphthalene dioxygenases, biphenyl dioxygenases, and monoaromatic dioxygenases (Baldwin et al., 2003). *Pseudomonas putida* has the most studied *nah* genes, which are encoded on the NAH7 self-transmissible plasmid, but the *nah* genes have also been studied in other organisms (Schell, 1986). The NAH7 plasmid contains the *nah* operon (*nahA-F*) necessary for the breakdown of naphthalene to salicylate, as well as the *sal* operon (*nahG-M*), which codes for enzymes to breakdown salicylate to TCA (Schell, 1986). The *nahR* is a regulatory gene found on the NAH7 plasmid that, when induced by salicylate, activates both the *nah* and *sal* operons (Schell, 1986).

1.6 Phytoremediation

Phytoremediation is defined as the use of plants to efficiently remove, detoxify or immobilize contaminants in the environment (UNEP, 2015). Plants can be used to assist in the remediation for a wide variety of contaminants including organic contaminants, metals, solvents, pesticides, explosives, and landfill leachate (Newman and Reynolds, 2004). There are a number of mechanisms associated with phytoremediation including phytoextraction, phytodegradation, rhizofiltration, phytostabilization, phytovolatilization and phytostimulation (Trapp and Karlson, 2001; UNEP, 2015). Phytoextraction begins in the roots and involves the absorption, translocation and storage of contaminants, often metals but in some cases organic contaminants. Phytodegradation occurs when the contaminant is taken up by the plant and then broken down, following uptake, through plant-mediated metabolic pathways. Phytodegradation, including rhizofiltration, can also be used to remediate contaminants in water. Rhizofiltration relies on the uptake or sorption of contaminants by the plant's roots (Trapp and Karlson, 2001; UNEP, 2015). Phytostabilization relies on the plant to immobilize or bind the contaminant into the soil matrix. Phytovolatilization occurs when the plant takes up, transforms and then volatilizes the contaminant to the atmosphere (UNEP, 2015). Phytostimulation is the use of plants to promote biodegradative activity of microorganisms within the rhizosphere, and is also known as rhizoremediation (Trapp and Karlson, 2001).

The phytoremediation techniques associated with organic contaminant removal, including diesel, are phytodegradation, phytovolatilization and rhizoremediation (UNEP, 2015). Both phytodegradation and phytovolatilization rely on the uptake of the organic contaminant. The uptake of organic contaminants is limited by the contaminants properties. Lipophilic compounds can cross the cell membrane more readily than polar compounds. However, if a compound is

very lipophilic it is likely to sorb to the roots, while a compound with intermediate lipophilicity is more likely to be transported to the upper regions of the plant. In addition to the properties of the organic compound, the availability of the compounds determines if the compound will be taken up by the plant. Should the compound be strongly attached to the soil matrix, its availability for uptake will be limited (Trapp and Karlson, 2001). Bioavailability of the contaminant can also be affected by the soil type (structure, texture, organic content), the moisture content of the soil, and temperature (Ansari et al., 2015). Once the organic pollutant has been taken up by the plant, phytodegradation will allow for the contaminant to be broken down into alcohols, acids, carbon dioxide, and water (Ansari et al., 2015). Should a contaminant that was taken up undergo phytovolatilization, the contaminant, or a modified form of it will be released into the atmosphere by the plant (UNEP, 2015).

1.7 Rhizoremediation

Rhizoremediation is the primary method by which petroleum hydrocarbons, such as diesel, are phytoremediated. The process relies on the interaction between plant roots and microorganisms that exist in the soil. Plant roots are capable of manipulating the soil environment through the release of root exudates, upon which microorganisms rely (Ansari et al., 2015). The plant is also able to improve both the physical and chemical properties of the soil, while increasing contaminant contact with the microbes. Ultimately, the combination of the plant and its associated microorganisms in rhizoremediation results in greater degradation than the other phytoremediation techniques (Kuiper et al., 2004). This is because processes like phytodegradation, phytovolatilization, and rhizofiltration can be limited due to the lipophilicity

of the contaminant and soil conditions, and the limited ability of plants to metabolize hydrocarbons.

The rhizosphere is the portion of soil and its associated microbial community that is affected by the root system of a plant (Barret et al., 2011). Rhizoremediation capitalizes on active zone plant-microbe interactions to stimulate microbial biodegradation of contaminants. There are three distinct areas that the rhizosphere can be divided into: the endorhizosphere, which is within the root tissue; the rhizoplane, which is the root surface; and the ectorhizosphere - the surrounding soil that is influenced by the root (Lynch, 1987 as cited in Badri and Vivanco, 2009).

Microorganisms and fungi that exist in the endorhizosphere are referred to as endophytes. Endophytes enter the plant through the root and are typically located in the root cortex or xylem. Endophytes generally do not negatively impact the plant; rather they assist the plant directly or indirectly. During phytoremediation, endophytes can play an important role in contaminant degradation as they may possess the necessary metabolic pathway to allow for more effective contaminant degradation. Plants are less likely to experience phytotoxicity from contaminants because of the endophytes' ability to degrade contaminants further (Weyens et al., 2009).

The ectorhizosphere is operationally defined as the soil that remains adhered to the root when a plant and its roots are removed from the ground and shaken. Within the ectorhizosphere a larger number of microorganisms can be supported through the release of various plant metabolites from live roots. The large number of microbes found in the rhizosphere, and their diversity, is the result of multiple factors: soil type, nutrients, moisture, pH, plant species and plant age (Grayston et al., 1997). Plants influence the soil environment by releasing organic compounds, which in turn influence the microbes present. The microbial community found

within the rhizosphere will differ from those found in bulk soil as a result of plant influences (Grayston et al., 1997). Root turnover, or root death, also stimulates microbes in the soil by providing dead root cell biomass for microbial utilization and by leaving behind air channels. Ultimately, the success of rhizoremediation relies on the establishment and survival of the plant, interaction of the plant and roots with microorganisms, and primary and secondary metabolism (Kuiper et al., 2004; Singer et al., 2004). Primary metabolites consist of carbohydrates, amino acids, organic acids, and fatty acids. The primary metabolites are heavily involved in the basic functioning of plants and are responsible for photosynthesis, respiration, plant growth and development, and hormone and protein synthesis. Secondary metabolites include sterols, alkaloids, glucosinolates, carotenoids, flavonoids and phenolic acids, which are important in plant defense (herbivores and microorganisms), plant signaling during stress, and the attraction pollinators (Hounsome et al., 2008).

1.8 Use of Native Species for Phytoremediation and Rhizoremediation

Early phytoremediation research focused on identifying plants that were capable of degrading contaminants or performing rhizostimulation. Researchers would commonly investigate and/or implement phytoremediation using these identified species, even if the species were not native to the target area. Although non-native species have been identified and found in laboratory settings to be capable of enhancing contaminant degradation, they cannot always be used for field applications. Non-native species in field applications can require greater management, additional irrigation, pesticide treatments, and fertilization (Nedunuri et al., 2010). Depending on the location, using non-native species can be a route for introducing invasive

species (DeVinny et al., 2015). Even if effective, non-native species are not able to lead to habitat restoration (DeVinny et al., 2015).

Native species can naturally revegetate a contaminant-impacted site or be planted, allowing for remediation and ecological restoration (Nedunuri et al., 2010). Species native to a region have become adapted to the conditions: temperature, precipitation, daylight, and soil characteristics (Frick et al., 1999). Typically, native plants are less expensive to use for remediation and ecological restoration (Nedunuri et al., 2010). However, it is possible for native species to struggle due to the harsh conditions found at a contaminated site (Nedunuri et al., 2010). This makes it important to identify native plants capable of growing under contaminated site conditions.

Ultimately, the establishment and survival of the plant is essential for the remediation of a site whether it is native or non-native (Nedunuri et al., 2010). For both native and non-native species, the addition of fertilizer and amendments, soil irrigation, and soil conservation practice can enhance the survival of plants (Nedunuri et al., 2010).

1.9 Root Exudates

Roots are well known for providing structural support and allowing the uptake of water and nutrients by plants but they have additional functions as well. Roots are capable of synthesizing, accumulating and secreting compounds known as root exudates. Root exudates are chemicals that have been released by roots into the surrounding soil and interact with the surrounding ecosystem (Walker et al., 2003). Root exudation involves the secretion of a multitude of compounds including ions, water, free oxygen, mucilage, enzymes, and primary and secondary compounds containing carbon (Bais et al., 2006). The rate of root exudation can vary as a result

of plant species and age, soil type, nutrient availability, microorganisms present in the environment, physical state of the plant, and root length (Badri and Vivanco, 2009; Bais et al., 2006; Hartmann et al., 2009). The process of secreting exudates taxes a plant's carbon supply; with up to 5-12% of a plant's photosynthetically created carbon, sugars, amino acids, and other metabolites being released through the roots (Huang et al., 2014).

Release of root exudates into the environment is known as rhizodeposition (Badri and Vivanco, 2009). Once exudates are released they can change the soil environment both physically and chemically, regulate the structure of the rhizosphere microbial communities and possibly act as messenger signals allowing the roots and microbes to interact (Walker et al., 2003). In addition, root exudates supply microorganisms with nutrients (carbon, nitrogen, phosphorous), organic acids, amino acids, sugars (Kuiper et al., 2004), and micronutrients (Miya and Firestone, 2001). Through the manipulation of the rhizosphere, root exudation can result in greater contaminant degradation in both soil and groundwater (Bais et al., 2009). It has been suggested that to further enhance the process of contaminant remediation, plant species could be matched to the desired microbial community that they are able to create with root exudation, for the targeted contaminant (Bais et al., 2009).

1.10 Role of Plant Secondary Metabolites in Rhizoremediation

Research looking into the potential role of individual plant secondary metabolites in rhizoremediation has increased over the years. Secondary metabolites are called such due to their low concentration in plants; they make up less than 1% of total carbon, and are considered to be non-essential to basic metabolic processes (Bourgaurd et al., 2001; Singer et al., 2003). Secondary metabolites are essential for plants to be able to adapt to their environments, as well as, interact with their surroundings (Bourgaurd et al., 2001). Functions of metabolites include

attracting or detracting insects and microorganisms, acting as antimicrobial agents, as stress response molecules, plant-to-plant signals, and germination and growth inhibitor (Singer, 2006).

Secondary metabolites typically contain one of the following structural backbones: alkaloid, isoprene, phenylpropene, and fatty acid/polyketide (Singer, 2006). Some secondary metabolites can enhance degradation of contaminants through microbial stimulation (Singer et al., 2003). One specific secondary metabolite compound researched is the organic acid salicylic acid (also referred to as salicylate). Salicylic acid is used to induce the systemic acquired resistance in plants and is an inducer of naphthalene dioxygenase gene expression (Singer et al., 2003). Naphthalene dioxygenase is an enzyme with fairly broad substrate specificity that catalyzes the cis-dihydroxylation of various aromatic compounds that can be found in diesel (Yi and Crowley, 2007, Chen and Aitken, 1999). Research performed by Chen and Aitken (1999) did show that when *Pseudomonas saccharophila* P15 was pre-incubated with salicylic acid, degradation of the PAH phenanthrene increased. However, at concentrations greater than 0.3 mM Chen and Aitken (1999) found that the removal of phenanthrene decreased. Yi and Crowley (2007), found that the addition of pure salicylic acid to soil had no effect on PAH degradation.

1.11 Fine Root Turnover

Fine roots are non-woody roots ≤ 2 mm in diameter that represent approximately 50% of a plant's total root system as well as the most active part of the root system (Rytter, 2013; Gunderson et al., 2008). Fine roots are the main source of water and nutrient absorption for plants (Rytter, 2013). These fine roots undergo cycles of growth and senescence; and the lifespan for fine roots can be anywhere from a week to multiple years (Rytter, 2013). Distribution of fine roots is influenced by surrounding environmental factors including moisture,

nutrient levels and soil temperature; these factors can determine the rate of turnover along with root tissue chemistry (Gunderson et al., 2008; Goebel et al., 2011). Roots have various concentrations of lignin, phenols, nitrogen and calcium within their tissue that are also related to the turnover rate (Goebel et al., 2011). It has been reported that when a resource or factor limits plant growth, it will in turn increase the lifespan of the fine roots (McCormack and Guo, 2014). Colder temperatures have been shown to result in a lower respiration rate, decreased mortality and increased root lifespan when compared to root systems of plants grown at warmer temperatures (McCormack and Guo, 2014). Limiting factors have also been shown in cases to increase root production, Gunderson et al. (2008) saw that the presence of hydrocarbon contamination resulted in greater fine root growth.

Once fine root turn over, microbial activity within the rhizosphere has been shown to increase, which has beneficial implications for rhizoremediation because of microbial stimulation (Rytter, 2013; Goebel et al., 2011; Singer, 2006). Through the turnover of fine roots, organic carbon is released into the surrounding soil, providing major influxes of carbon (Rytter, 2013; McCormack and Guo, 2014). In addition to the release of organic carbon, root turnover releases secondary metabolites into the rhizosphere, as well as oxygen via air channels left behind by roots. This can result in a change in the microbial community by selecting for microorganisms and/or altering catabolic and metabolic status (Martin et al., 2014). Oxygen released into the rhizosphere can enhance the activity of mono- and dioxygenase enzymes resulting in aerobic degradation (Leigh et al., 2002). Leigh et al. (2002) found that contaminants in the soil were reduced through sustained rhizoremediation that resulted from root turnover and the release of secondary compounds.

1.12 Advantages and Disadvantages of Phytoremediation

Phytoremediation offers many benefits for the removal of diesel from the environment. The cost of implementing phytoremediation is lower than that for removing and incinerating contaminated soil; the cost of maintaining the operation is also low (Gerhardt et al., 2008). Typical *ex-situ* methods can cost as much as $200-1,500 per ton of soil while plants implemented for phytoremediation can cost $10-50 per ton of soil (Pilon-Smits et al., 2006; Schnoor, 2008 as cited in Gerhardt et al., 2008). Anywhere that plants can physically grow, they can be used for remediation, and there is no restriction based on the size of the contaminated area (Gerhardt et al., 2008). Plant roots can stabilize soil, helping to prevent erosion from wind and water along with adding nutrients, oxygen, and organic materials. Roots are also are able to increase the bioavailability of diesel trapped in micropores, which otherwise would remain sequestered (Gao et al., 2011). Plants may be unbiased towards contaminants and this gives some the ability to remediate diesel along with any occurring co-contaminants. It is best to use local plant species when remediating a site; they are more likely to survive and can also serve to reconstruct the natural habitat (Gerhardt et al., 2008).

Plants and their roots offer the ability to enhance diesel removal by increasing both abiotic losses and microbial degradation through biostimulation (Sun et al., 2010). Phytoremediation can result in the uptake of diesel (Gao et al., 2011), which can be sequestered or enzymatically broken down and phytovolatilized, however these processes result in less extensive diesel removal or degradation than rhizoremediation, which involves microbial processes (Gerhardt et al., 2008).

There are certain disadvantages associated with using plants that cannot be overcome; for instance, some plants, especially trees, take a long time to grow and fully establish on a site.

Even after vegetation is established on a contaminated site and after remediation is complete, there could be problems with the disposal of plants if they are contaminated. Some types of contaminants are problematic for phytoremediation. For instance, should the contaminant be readily taken up and stored in the plant, it potentially could enter the food chain (DeVinny et al., 2015). Should a site be heavily contaminated, it can negatively impact plant growth and stress hormones may be released, such as ethylene, decreasing plant growth (Gerhardt et al., 2008). There are a number of environmental stressors including temperature, precipitation, or herbivory that cannot always be controlled in the field (Gerhardt et al., 2008). Soil conditions may not always be conducive to plant growth, and may require amendments such as fertilizer or additions that improve either drainage or water-holding capacity, which can increase the costs of successful implementation.

Laboratory methods and practices do not always transition well into the field because certain factors are not, or cannot be, taken into consideration in the lab. Experiments conducted in the lab using fresh diesel are not fully representative of field conditions, where weathered diesel might be encountered. Hot spots create issues as well, in which soil contamination levels throughout a site are not homogeneous, and some spots contain more contamination than others (Gerhardt et al., 2008). Hot spots require care in sampling strategies when monitoring remediation, as they can skew results, and hot spots can affect plant health. Because of the hydrophobicity of diesel components, diesel compounds can sorb to roots, resulting in the appearance that phytoremediation is not having any impact on diesel removal or a false positive indication of diesel loss.

1.13 Research Needs

Research in phytoremediation has advanced significantly over time, yet several important challenges remain. It is time to look into phytoremediation areas such as: extreme conditions (drought and frost), weathered contamination, and mixed plants communities; all conditions likely to be encountered in the field. In laboratory studies there has not been a wide range of conditions researched because they are often difficult to replicate. In addition to extreme conditions needing further research, the native plants that grow in these regions, which are capable of enhancing contaminant degradation, need to be considered. Many studies concentrate on plants that grow in temperate climates and tend to focus on the same species repeatedly, thus limiting our knowledge. These are many items that need to be addressed in the lab, but there are also items that need to be tested in the field. Researchers often forgo field studies as they require more time.

Phytoremediation is not a new topic, but it is now time for the knowledge to grow regarding the more specific associated methods. It is well known that rhizoremediation is the key process in petroleum degradation via phytoremediation, however what specifically enhances this process needs more attention. The events that occur in the rhizosphere involve a series of complex interactions between microorganisms, roots, plant compounds, and environmental conditions. The roots and the plant compounds they release, specifically during root turnover, need additional research. There are over 100,000 secondary plant metabolites and their effect in the environment is not fully understood. Salicylic acid has shown promise but only a few papers on its effects on rhizoremediation have been published, more work is needed to investigate this and other plant secondary metabolites.

Besides researching the methods and conditions under which phytoremediation occurs, researchers need to look at where phytoremediation can be applied successfully. It is well known that phytoremediation is a cost effective method for contaminant degradation. More studies need to investigate how phytoremediation can be implemented in rural and under-developed communities that have limited means for *ex-situ* remediation. Communities may benefit from implementing field studies in these rural locations as well, through local engagement, educational, and economic opportunities. Now is the time for researchers to fill in these data gaps.

1.14 Conclusions from Literature Review

Alaska is a unique state. It is the largest in the United States and has over 300 remote communities not connected to a road system. Alaska also has a wide variety of environments, including sub-arctic and arctic conditions. Winter conditions are extreme and remote communities rely heavily on diesel, as such there have been multiple diesel spills ranging from drips and drops to thousands of gallons. It is not possible to ship out the contaminated soil from these communities, and without assistance natural attenuation is slow. Phytoremediation may be a beneficial technology to be implemented in these communities. Willows grow readily within the sub-Arctic and Arctic and have been shown to increase contaminant degradation. In addition, willows are known to contain salicylic acid, a secondary plant metabolite that holds promise for increasing rhizoremediation. Willows may prove valuable for enhancing the rhizoremediation of diesel contamination in remote Alaskan villages.

Section 2 Materials and Methods

2.1 Kaltag Site Description

Kaltag, AK, lies along the western bank of the Yukon River at the base of the Nulato Hills, 335 miles west of Fairbanks, AK. Kaltag is a municipality and has a Bureau of Indian Affairs (BIA) recognized traditional council; many of the 184 residents are Koyukon Athabascan. During the summer months, temperatures average 21 °C, while in the winter months the average is -12 °C or below; annually the area receives 41 cm of precipitation and 188 cm of snowfall (http://commerce.state.ak.us/cra/DCRAExternal/community/Details/1c478003-280c- 46e8-b893f98ffa651efe). Local soils are primarily composed of interbedded silt, sand, and gravel. Permafrost varies throughout the area and can exist as shallow as 1.1 m below ground surface (bgs); the active layer above the permafrost is more readily impacted by environmental factors. Groundwater exists at about 1.2 m bgs (Guritz, 2011).

The first aboveground storage tanks (AST) were installed by the BIA in 1960, east of Kaltag School (Figure 3). The BIA tank farm was later replaced when the Yukon-Koyukuk School District (YKSD) developed its own tank farm in 1976. The YKSD tank farm consisted of four ASTs placed on top of a string reinforced polyethylene visqueen liner which was covered with gravel. Between 1984 and 1985 the YKSD tanks were plumbed into the Alaska Village Electric Cooperative (AVEC). The setup for this tank farm involved seven vertical ASTs and two horizontal ASTs with a fuel capacity of 28 – 34 m^3; these tanks were also installed on a string reinforced polyethylene visqueen liner that was covered with gravel. Between 1996 and 1998 the YKSD tanks were replaced by a single day tank (a single distillate fuel tank used to fuel generators or boilers) and by 2002, the AVEC tanks were decommissioned and removed from the area (Guritz, 2011; Guritz, 2012).

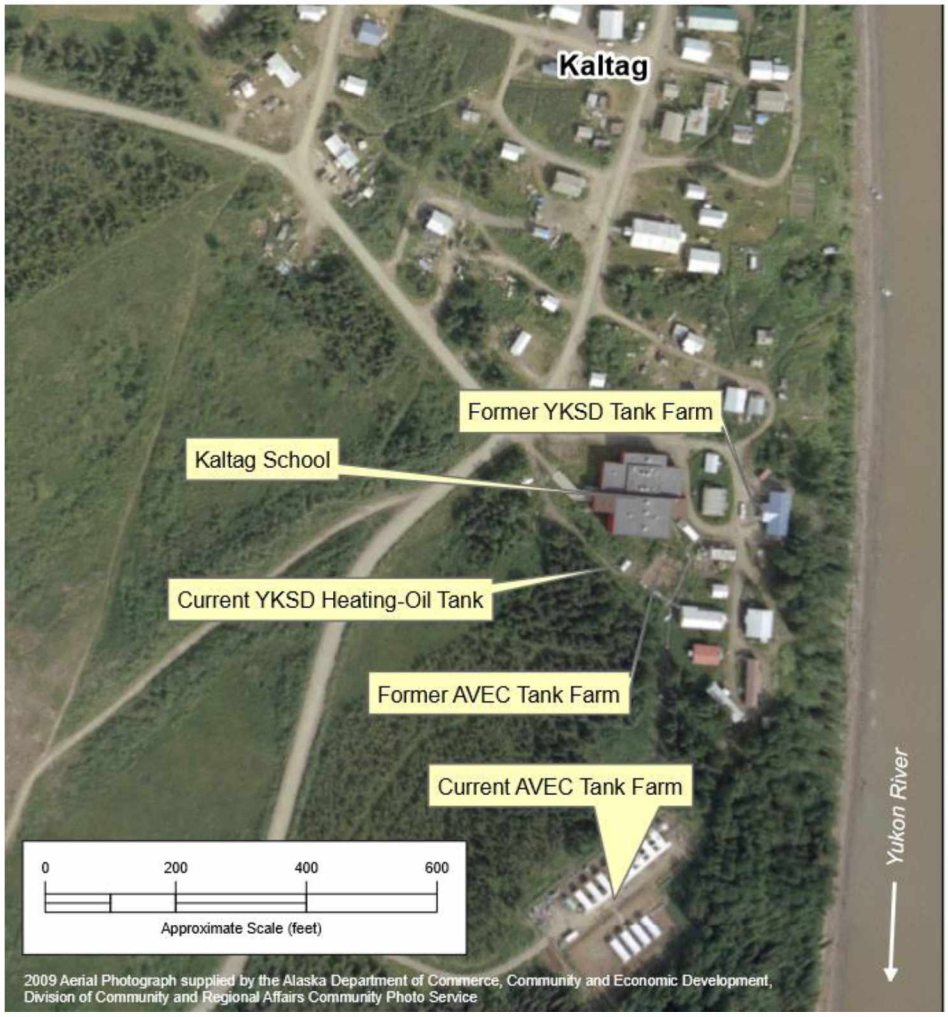

Figure 3: Kaltag, AK Fuel Tank Aerial View

Over the years numerous spills, overfills, and pipe leaks have occurred at each of the tank farms. The most common reasons for such the release of diesel fuel in Kaltag, AK are believed to be operator error and aging equipment. In 1991, Kaltag, AK reported to the ADEC that there was a visible petroleum sheen on melt waters at the Kaltag School and surrounding areas. ADEC contracted site characterizations and assessments that occurred over multiple years, as

well as interim corrective actions. Corrective actions consisted of removing contaminated soil off-site, roadway-seepage control, and seepage filtration systems (Guritz, 2012).

Early assessments indicated that diesel range organics (DRO) were present at concentrations above ADEC cleanup levels. A site assessment performed in 1998 found a large oil stained area at the former tank farm adjacent to the school. The staining was no longer visible by 2007, but there was limited vegetation growth in the area (Guritz, 2011). While performing site assessments at Kaltag, AK, it was found, through speaking with Kaltag residents, that there were other sources of contamination within the area that would need to be investigated further: a damaged above-ground fuel line system that supplied the YKSD boiler and a heating oil transfer tank that was used to fill individual heating oil tanks with a rubber hose and spigot (Guritz, 2011).

In 2010 Shannon & Wilson, Inc. (SWI) visited Kaltag, AK to assess the overall extent of DRO contamination. The highest levels of DRO contamination were found at the heating oil transfer tank (75,400 mg/kg), former BIA tank farm (58,900 mg/kg), fuel line leaks (47,400 mg/kg), YKSD tank farm (20,300 mg/kg), and former AVEC tank farm (7,170 mg/kg) (Guritz, 2011).

2.2 Soil Collection

Soil was collected from Kaltag on June 27 and 28, 2012, prior to excavation activities. Contaminated soil was collected from 64° 19.590'N, 158° 43.379'W, next to the YKSD shop heating oil transfer tank (Figure 4), which was identified as contaminated based in a 2011 Site Characterization Report submitted to ADEC by SWI (Guritz, 2011). Uncontaminated soil was collected from the playground at N 64° 19.590' W 158° 43.714'. Both the contaminated and clean soil samples were collected to a depth of 15 cm bgs using hand tools. Samples could not

be collected any deeper because of frozen organic soil. All samples were placed in buckets. Buckets were placed in larger containers filled with ice packs while being transported from Kaltag, AK to the University of Alaska Fairbanks (UAF), where they were then stored at 4 ºC for about 1 year. Weathered-diesel loss during that 1 year period was minimal; loss was determined by comparing gas chromatography/mass spectrometry (GC/MS) chromatograms shortly after soil collection and with chromatograms immediately prior to the start of the study. Both weathered-diesel contaminated and clean soils collected from Kaltag, AK were a silty loam mixture with low organic content. Organic content was determined by liquid chromatography at UAF.

Figure 4: Sample Collection Point

2.3 Microcosm Studies

Microcosm studies were used to assess the intrinsic biodegradation rate of weathered and fresh (spiked) diesel in soil from Kaltag, AK, and to compare the effects of willow roots, salicylic acid, and fertilizer addition on biodegradation rates. Microcosms were set up in 0.24 L canning jars that were autoclaved prior to use. Jars to be used for microcosm incubation at 4 °C were pre-chilled at 4 °C for 48 hours prior to the addition of soil.

All soil, contaminated and clean, was passed through a 2 mm sieve and stored at 4 °C until the start of the experiment. Soils to be incubated at 20 °C were brought to temperature 48 hours in advance to decrease the possibility of a delayed response of the microorganisms. Soil was sterilized for control incubations by autoclaving clean soil collected from Kaltag, AK. Clean soil spiked with fresh diesel was prepared by individually adding diesel to each microcosm and mixing the soil on a vortex mixer for 2 minutes. The final concentration for microcosms spiked with fresh diesel was 3,000 mg/kg. The amount of diesel added was selected with the goal of targeting soil concentrations likely to be used for phytoremediation. Trapp and Karlson (2001) found that diesel at a concentration of 1,000 mg/kg decreased plant transpiration but was not lethal, while diesel at a concentration of 10,000 mg/kg had a significant negative impact on willows. Microcosms used for respiration studies were filled with 15 g of soil and those for biological and chemical analysis contained 85 g.

Amendments were added to microcosms as shown in Table 1, which summarizes the experimental design. A complete list of microcosms and their parameters is found in Appendix A. Fertilizer of the type 20-20-20 (N-P_2O_5-K_2O) was crushed, dissolved in water and added to soils to achieve a concentration of 500 mg fertilizer/kg soil. Salicylic acid was also dissolved into water prior to adding to soil at a 1% w/w ratio. Fine willow roots were collected from a

stand across from the Fairbanks International Airport, 64°47'40"N, 147°53'45"W. Crushed willow root was added to microcosms at a 1% w/w ratio; prior to adding, fine willow roots were frozen with liquid nitrogen and crushed. The soil moisture content was adjusted, as needed, by adding appropriate levels of water to microcosms to account for the addition of amendments that were dissolved in water. The water adjustment ensured that all soil microcosms had the same moisture content at Day 1.

Table 1: Microcosm Experimental Design

	Weathered Diesel-Contaminated Soil	Fresh Diesel-Spiked* Clean Soil	Clean Soil	Fresh Diesel-Spiked* Clean, Sterilized Soil
Control (No Amendments)	4 Jars	4 Jars	3 Jars	2 Jars
Fertilizer (Fert.)	4 Jars	4 Jars	3 Jars	2 Jars
Salicylic Acid (SA)	4 Jars	4 Jars	3 Jars	2 Jars
SA & Fert.	4 Jars	4 Jars	2 Jars	2 Jars
Crushed Willow Root (WR)	4 Jars	4 Jars	2 Jars	2 Jars
WR & Fert.	4 Jars	4 Jars	3 Jars	2 Jars
Total Number of Jars	**24 Jars**	**24 Jars**	**16 Jars**	**12 Jars**

*Spike soil to concentrations of 3,000 mg/kg diesel

Once all microcosms were prepared, i.e. after addition of any amendments and/or diesel, they were stored at their respective incubation temperatures for 12 hours to allow soils to adjust to their respected temperatures. Microcosms were incubated at 20 °C (room temperature) and at 4 °C to determine the effects of temperature on biodegradation. Experiments were performed over a 90 day period to simulate a summer growing season.

Samples were collected from microcosms designated for biological and chemical analyses on Days 1, 14, 45, and 90, microcosms were not sacrificed during sampling events. Methods used to determine the biodegradation rate of diesel included respirometry, chemical and biological analyses through GC/MS, most probable number (MPN) counts of DDM, and the Microtox® assay to assess soil toxicity. Gravimetric moisture calculations were performed to monitor soil moisture over the course of the experiment.

2.4 Diesel Concentration Analysis by GC/MS

GC/MS analysis was used to determine the total DRO concentrations in samples. Extraction and sampling procedures were developed using the AK Method 102 (ADEC, 2002). Soil samples were collected from microcosms using a clean-metal spatula to grab soil from various locations and depths within the microcosm to develop a composite sample. Prior to reusing a metal spatula, the spatula was rinsed in an alcohol solution and then sterilized over a Bunsen burner flame. For every 20 samples, a duplicate sample was collected from the microcosms by dividing the contents of the jar in half. Samples that could not be extracted immediately after collection were placed in polypropylene centrifuge tubes at stored at -80 °C. Samples consisting of 10 g of soil were placed in 250 mL glass flasks for immediate extraction. Each sample was spiked with 250 µL of the surrogate naphthalene-d8 immediately before extraction with 25 mL methylene chloride. Flasks were placed on a rotary table at a speed of 150 revolutions per

minute (rpms) for 1 hour. Subsequently, methylene chloride extract samples were collected with a glass syringe and placed in amber glass vials with a Teflon-lined septa lid.

Samples were analyzed using an Agilent Technologies 6890N Network GC System coupled to a 5873 Mass Selective Detector. One and a half mL of each extract was placed in a GC vial and was spiked with 25 µL of 0.1% pure d5-nitrobenzene as an internal standard. A calibration curve was developed using diesel calibration standards of 1,500 mg/kg, 1,250 mg/kg, 1,000 mg/kg, 625 mg/kg and 312.5 mg/kg. Calibration standards were run once per day. A continuous calibration verification (CCV) standard of 5,000 mg/kg diesel containing the internal standard and surrogate was run every 20 samples. In addition, two reagent blanks each containing 1.5 mL methylene chloride spiked with 25 µL of naphthalene d-8 and 25 µL of d5-nitrobenzene were analyzed every 20 samples. Samples were run through a 5% phenol column with a length of 30 m, diameter of 0.32 mm, and a film of 0.25 µm. A 10:1 split injection method was used with a flow of 36.5 mL/min. Agilent MSD Chemstation E02.00 software was used to identify specific peaks and to manually integrate peaks.

2.5 Enumeration of Diesel Degrading Microorganisms

An MPN method adapted from Haines et al. (1996) was used to quantify the abundance of indigenous diesel-degrading bacteria in soil samples collected from the microcosm incubation study. Three 1 g samples were collected using a clean-metal spatula to grab soil from various locations and depths within each microcosm and combined to create a composite sample. The metal spatula was sterilized before each use; the spatula was rinsed with an alcohol solution and heated above a Bunsen burner flame. Samples were placed in polypropylene centrifuge tubes that contained 4 g of sterile glass beads. To each tube 10 mL of 1% w/v sodium pyrophosphate

($Na_4P_2O_7$) was added. The centrifuge tubes were then placed on their side on a rotary platform shaker set at 150 rpm for 1 hour. After 1 hour of mixing, tubes were placed upright and the suspension was allowed to settle for 30 minutes.

Sterile Bushnell-Haas medium was added to 96-well plates, 180 µL per well. From the centrifuge tubes, 20 µL of microbial suspension was pipetted to the first column of the 96-well plate and mixed thoroughly within each well. Following this, 20 µL of solution from the first well was pipetted to the adjacent well (next column), creating a ten-fold serial dilution of each sample across the plate. Every 96-well plate (Figure 5) contained three replicates per sample.

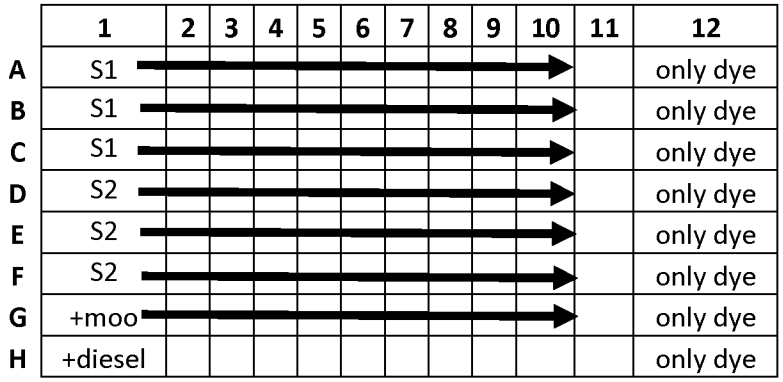

Figure 5: 96-Well Plate Setup for MPN of Diesel Degraders

Row G is a false-positive control well that contains microbes and no diesel, a positive result should not occur due to the absence of hydrocarbons. Row H is a false-negative control that only contained diesel fuel and Bushnell-Haas medium, no microbes. After addition of the microbial

suspensions, 5 µL of diesel fuel (carbon source) was added to all wells except for the false-positive control wells (Row G). Diesel fuel was filtered using a 0.22 µm filter prior to addition.

All 96-well plates were covered with lids and then sealed using Parafilm. Subsequently, the plates were sealed in Ziploc bags and placed in a dark location at room temperature for 14 days. Following the incubation period, 50 µL of 3 $g \cdot L^{-1}$ Sigma Aldrich p-Iodonitrotetrazolium (also referred to as INT dye) was added to each well after being filtered with a 0.22 µm filter. The p-Iodonitrotetrazolium is an indicator dye that forms a red/pink color when microorganisms are actively respiring. After the addition of the p-Iodonitrotetrazolium, the 96-well plates were resealed and placed back in the dark at room temperature for 48 hours. Positive wells were identified at the end of the 48 hours and results were calculated using published MPN tables (Man, 1983).

2.6 Respiration Analysis

A LI-COR 6262 near-infrared detector was used to determine fluxes of CO_2 using a LI-COR IRGA coupled with a flow-through cap that fits the opening of a 0.24 L jar (Figure 6). Once a week, the LI-COR 6262 instrument was calibrated using 1,000 ppm CO_2. For analysis, 4 °C microcosms were maintained as close to the incubation temperature as possible using ice packs. To purge the headspace, microcosms were aerated in front of a fan for 10 seconds before readings were collected. Carbon dioxide readings were collected by the LI-COR 6262 every 3 seconds for a total of 2 minutes for each microcosm at each time point. Following the respiration analysis, microcosms were allowed to re-aerate by setting the loose lids on top of each jar for 1 hour to prevent microcosms from becoming anaerobic.

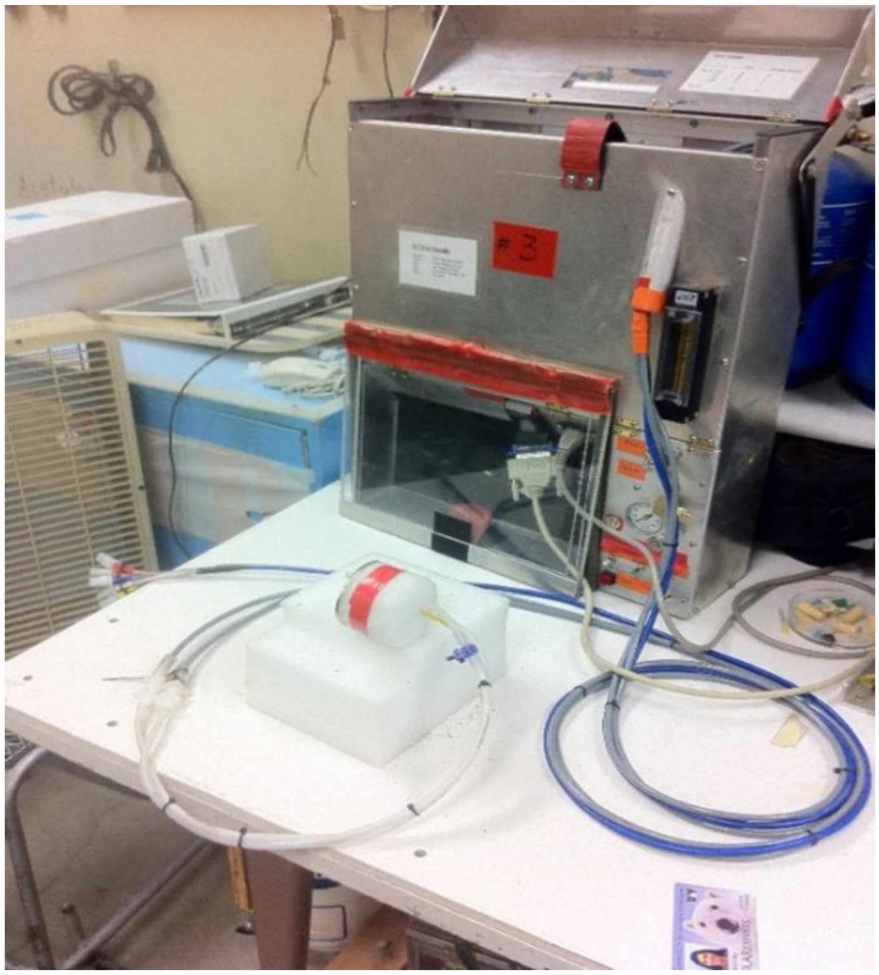

Figure 6: LI-COR 6262 Setup for Respirometry

Respiration analysis was performed for all microcosms dedicated to respirometry studies. All 20 ºC microcosms were analyzed on Days 1, 3, 6, 9, 14, 20 and then every seven days until Day 90. Samples for Day 14 were collected, but are not recorded in the results. This is because on Day 14 an alternate sampling method was attempted, this alternate method did not produce results. In 4 ºC microcosms, respiration was measured every 7 days until Day 91. A different time course for 20 ºC and 4 ºC was selected because we anticipated a quick spike in respiration

that would then decrease for soils incubated at 20 °C, whereas at 4 °C, we expected CO_2 production to be slower and to show a peak in respiration later in the experiment, if at all.

2.7 Microtox® Analysis

The Microtox® assay is an in-vitro toxicity test that relies on the bioluminescence of *Vibrio fischeri*. When *V. fischeri* is exposed to toxins, the metabolic pathway that controls the luminescence is disrupted and prevents further bioluminescence. Reduction in bioluminescence response due to acute toxicity is quantified using a Microtox® M500 self-calibrating temperature-controlled photometer. Soil samples were analyzed using the Basic Solid Phase Test (BSPT) protocol, which allows for solid samples to be tested utilizing serial dilutions. For this test, samples are placed in an aqueous suspension allowing the microorganisms to come into direct contact with the solid sample. Seven grams of soil were collected from various locations and depths within each microcosm, using a clean-metal spatula, to create composite samples. The metal spatula was sterilized in between uses using an alcohol rinse and heated over a Bunsen burner to prevent cross-contamination. Samples that were not immediately analyzed were stored in polypropylene centrifuge tubes at -80 °C until Microtox® analysis.

For the analysis, cuvettes were placed into the temperature controlled incubator wells, as specified by the Microtox® BSPT, as well as the reagent well. Cuvettes in rows A, C, and E (Figure 7) were filled with 1 mL diluent, except for the cuvette that would contain the soil diluent mixture (well 1A). The diluent is a 2% NaCl solution that provides osmotic protection for the *V. fischeri* (AZUR, 1998). The cuvettes in rows B, D and F (Figure 7) were filled with 0.5 mL of diluent. The cuvette within the reagent well was filled with 1 mL of reconstitution solution.

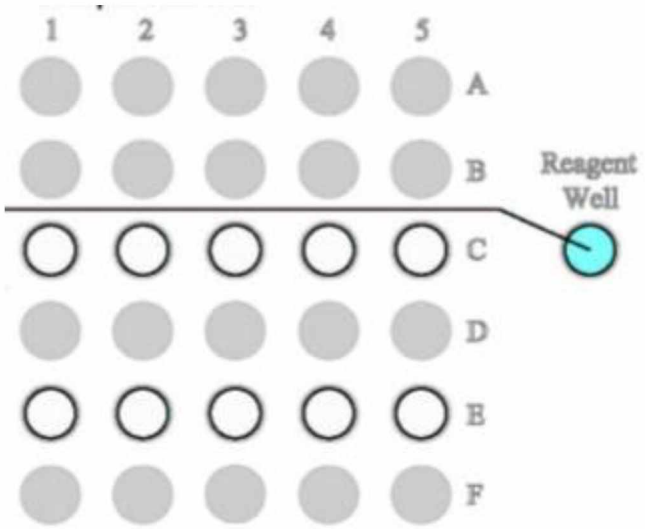

Figure 7: Microtox® Setup (AZUR, 1998)

Once soil samples were thawed, they were placed in 50 mL beakers and 35 mL of diluent was added. Beakers were placed on magnetic stir plates and a magnetic stir bar was used to mix the solution for 10 minutes; vortex depth was kept at 50% of the liquid level. Subsequently, 2 mL of the soil diluent mixture was pipetted into the starting cuvette (well 1A).

A 1:2 serial dilution was created by pipetting 1 mL of the soil diluent mixture into the adjacent cuvette, mixing the solution with the pipette prior to each transfer. Dilutions proceeded through rows A, C, and E (Figure 5); from A2 1 mL of solution was discarded as specified by the Microtox® BSPT. Following the serial dilutions, there was a 5 minute period of waiting. One vial of Microtox® acute toxicity reagent was reconstituted using the reconstitution solution in the reagent well. The 1 mL of reconstitution solution was added to the reagent vial, swirled for 4 times, and then poured back into the cuvette. Reconstituted bacteria were used for a maximum of 4 hours.

Ten microliters of reagent, containing the reconstituted *V. fischeri*, were pipetted into cuvettes in rows B, D, and F (Figure 7), followed by a 15 minute waiting period. The first cuvette was placed in the READ well, set, and then light levels analyzed for a zero time result (I_0), this was done for all cuvettes in rows B and D (Figure 7). Immediately after the time zero readings, 0.5 mL of solution from cuvette in A1 was added to that in B1 (Figure 7) and mixed. These same steps were performed for cuvettes in A2 and B2, A3 and B3,...A5 and B5 (Figure 7). The exact same steps were taken for cuvettes in Rows C and D, and Rows E and F (Figure 7). Light levels were then read from cuvettes in rows B, D and F (Figure 7).

Due to the cost of the Microtox® analysis, only a limited set of samples was analyzed, specifically contaminated Kaltag soil at 20 °C from Day 1 and Day 90, as well as contaminated Kaltag soil at 4 °C on Day 1.

2.8 Soil Moisture Content Analysis

The moisture content of soil within microcosms was determined gravimetrically on Day 1 and Day 45 using the ASTM D4959 method. Three grams of soil were collected using a clean-metal spatula to grab soil from various locations and depths in each microcosm and combined to create a composite sample. Prior to each use, the metal spatula was sterilized using an alcohol rinse followed by heating over a Bunsen burner flame. Samples were placed in heat resistant dishes and the total weight was measured. Soil and containers were placed into a 105 °C pre-heated oven equipped with a vapor hood located above for 1 hour. Dishes were then weighed; the process was repeated until there was no longer any change of weight. Once this point was reached, the moisture content was calculated based on the difference between soil wet and dry weight. Results of gravimetric analysis are found in Appendix B.

2.9 Statistical Analysis

Multivariate statistics can be useful to analyze large, complex data sets, e.g. in biological systems (McGarigal et al., 2000) by using an empirical descriptive statistics approach that allows for the optimal combination of variables and simplifies the data set so that important parameters can be isolated from noise (Harris, 2001). Further detail on multivariate statistics can be found in Appendix C.

Partial least squares (PLS) regression is a multivariate analysis approach to predict dependent variables from a large set of independent variables (**X**), based on identification and extraction of latent independent variables (orthogonal factors or principal components) that have the highest predictive power. Thus latent variables (underlying factors that are not directly observed) are used to model the relations between observed variables (Rosipal and Krämer, 2006; Tobias, 1997). PLCS can therefore be seen as a combination between multiple regression and principle component analysis. PLS is a soft modeling approach that can be used when theoretical knowledge is limited and distributional assumptions are not applicable (Lohmöller, 1989). Further detail on multivariate statistics can be found in Appendix D.

In the current study, a non-linear iterative partial least squares (NIPALS) algorithm was utilized for PLS regression modeling employing the Unscrambler® software. The algorithm proceeds by extracting one factor, also known as a PC, iteratively through repeated regressions of **X** (example shown in Appendix E) on scores $\hat{t}$ to obtain an improved $\hat{p}$. Repeated regressions of **X** on the $\hat{p}$ to obtain improved $\hat{t}$ are performed as well. Table 2 should be referred to for the NIPALS algorithm.

Table 2: NIPALS Algorithm Symbol Definition

Symbol	Meaning
X	x-value for a vector or matrix
$\hat{t}$	Predicted value scores of a vector or matrix
$\hat{t}'$	Derived value scores of a vector or matrix
$\hat{p}$	Predicted x-loadings of a vector or matrix
$\hat{p}'$	Derived x-loadings of a vector or matrix
$\bar{x}'$	Derived x-value mean
a	Factor (PC) number, number of Factors (PCs)
$\hat{\tau}_a$	Eigenvalue
$\hat{\tau}'_a$	Derived Eigenvalue

The NIPALS algorithm pre-scales the **X** variables to ensure noise levels are comparable. **X** variables are centered by subtracting the calibration means $\bar{x}'$ resulting in x_o. The calibration equations are used to determine how well the model describes the collected data (CAMO, 2016). Then for the PCs $a = 1, 2....A$ and using X_{a-1} both $\hat{t}_a$ and $\hat{p}_a$ are computed.

To start, the NIPALS algorithm selects $\hat{t}_a$ equal to the column in X_{a-1} that has the highest remaining sum of squared errors between predicted and reference values. Steps 1-5 are then repeated until convergence is reached:

1) $\hat{p}_a = (\hat{t}'_a \hat{t}_a)^{-1} * \hat{t}'_a * \mathbf{X}_{a-1}$ This equation is used for the loading vector $\hat{p}_a$ improved estimate through the projection of the matrix $\mathbf{X}_{a-1}$ on $\hat{t}_a$
2) $\hat{p}_a = \hat{p}_a * (\hat{p}'_a \hat{p}_a)^{-.05}$ to avoid ambiguity the scale length of $\hat{p}_a$ to 1.0

3) $\hat{t}_\alpha = X_{\alpha-1} * \hat{p}_\alpha (\hat{p}'_\alpha \hat{p}_\alpha)^{-1}$ this equation is used for improving the $\hat{t}_\alpha$ estimate score for this factor through the projecting matrix $\mathbf{X}_{\alpha-1}$ on $\hat{p}_\alpha$

4) $\hat{\tau}_\alpha = \hat{\tau}'_\alpha * \hat{\tau}_\alpha$ equation for the improved estimate of the eigenvalue $\hat{\tau}_\alpha$

5) Convergence is checked. If $\hat{\tau}_\alpha$ minus $\hat{\tau}_\alpha$ determined through the previous iteration is smaller than a pre-specified constant (e.g. 0.0001) multiplied by $\hat{\tau}_\alpha$, convergence is reached. If the method has not converged the process begins at step 1

Once convergence is reached, the following equation is used to subtract the effect of the factor $\mathbf{X}_\alpha = \mathbf{X}_{\alpha-1} - \hat{t}_\alpha * \hat{p}_\alpha$. The process then begins again with the next factor until all factors have been processed by the NIPALS algorithm. This allows us to determine how the factors are based upon the fixed variables, and in what combination the factors influenced the results (ex. percent DRO loss). The PLS method was applied to overcome the lack of sample replicates. It uses a limited information approach; as such it assumes nothing about the scale or population of measurements and works without assumptions of distribution (Haenlein and Kaplan, 2004).

Section 3 Results

3.1 Diesel Range Organics Analysis Results

GC/MS measurements were performed for all weathered diesel-contaminated microcosms; those had an average initial diesel concentration of 55,000 ± 11,000 mg/kg. Diesel concentrations on Day 1 and Day 90 from weathered diesel-contaminated microcosms and from fresh-diesel spiked microcosms are reported in Appendix F. Over the course of incubations, warmer temperatures (20 °C) resulted in greater DRO loss than colder temperatures (4 °C), as shown in Figure 8 (a table of DRO loss percentages from weathered-diesel microcosms is in Appendix F). Results shown in Figure 8 are the average of duplicate microcosms. Figure 8 not only shows that warmer temperatures resulted in greater DRO loss, but that the addition of crushed fine willow root and fertilizer at both 20 °C and 4 °C showed greater DRO loss (76.6% at 20 °C and 43.2% at 4 °C).

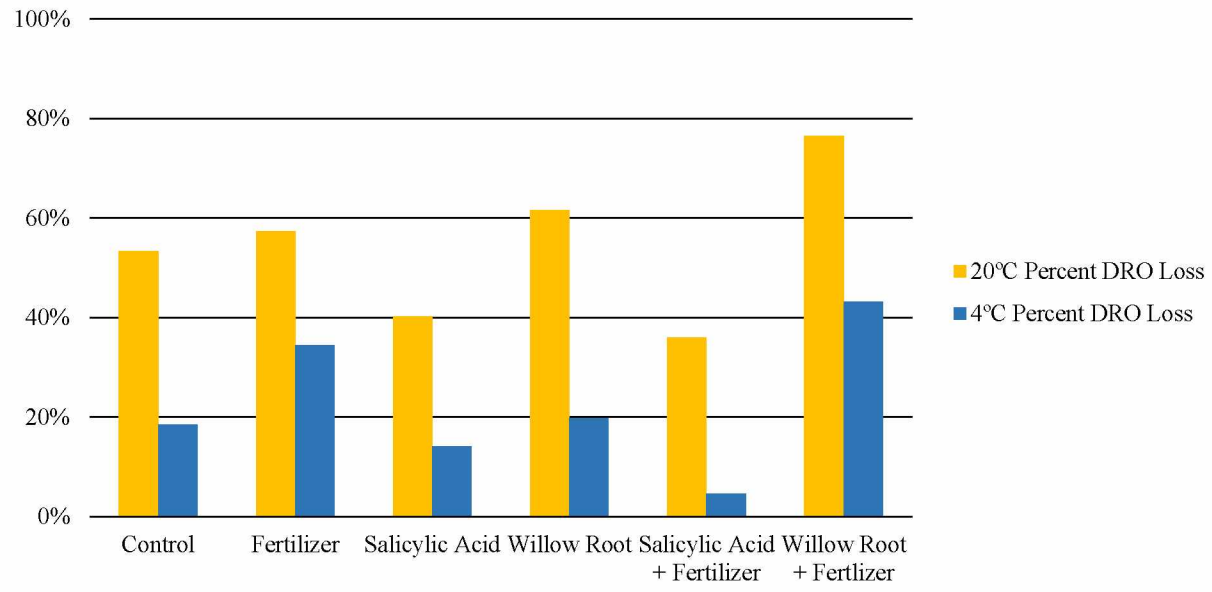

Figure 8: Total DRO Loss from Weathered-Diesel Microcosms Over 90 Days

Results also showed that the addition of pure salicylic acid, even in combination with fertilizer, decreased DRO loss at both temperatures (36% at 20 °C and 4.7% at 4 °C). Results from fresh-diesel spiked GC/MS analysis are in Appendix F.

PLS regression analysis was performed for the different treatments (temperature, fertilizer, salicylic acid and willow root) of the weathered diesel-contaminated soil microcosms. Through the PLS method, it was determined whether relationships existed that correlated with the loss of diesel observed in microcosms. Weathered-diesel contaminated results were reasonably well modeled by PLS analysis as shown by the regression graph in Figure 9 and Figure 10. Figure 9 indicates that 2 factors (i.e. principle components) were capable of explaining the variance. The validated explained variance was similar to the calibrated explained variance, which demonstrates that the model is reliable. After the first two factors, the PLS regression is modeling noise: this is indicated by little increase in explained variance with the addition of each factor. The regression graph in Figure 10 shows the ability of the PLS method to model the response variable, in this case the DRO removal percentage over 90 days. The R^2 value of the calibrated data was 0.8369 and the R^2 value of the validation data was 0.7683. The R^2 value measures how well the data fits to a regression line, 1.00 or 100% would mean that the model is able to fit all of the data. At 0.00, or 0% the model would be unable to fit the data. Figure 10 also shows close results between the validated and calibrated explained variances, which supports the model as reliable.

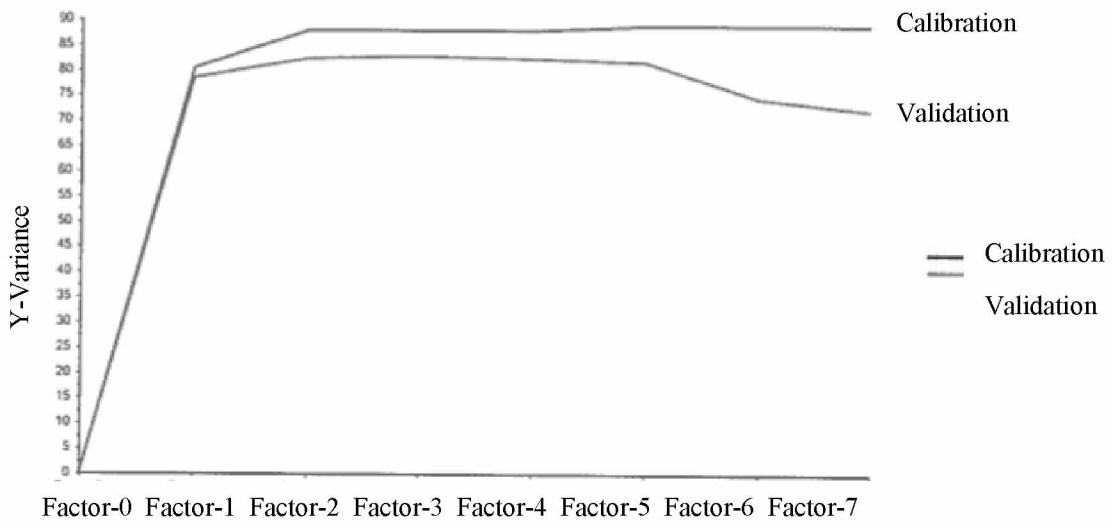

Figure 9: PLS Factors Required to Explain DRO loss from Weathered-Diesel Microcosms

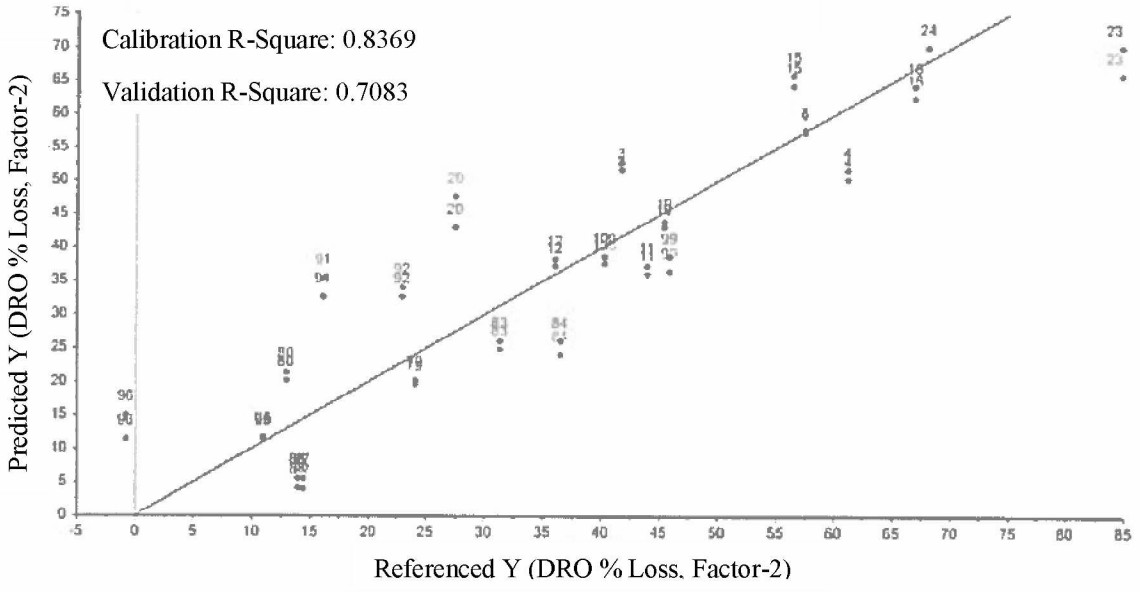

Figure 10: PLS Regression Modeling of DRO Loss from Weathered-Diesel Microcosms

The correlations loading figure (Figure 11) shows that Factor 1 (x-axis) is predominantly temperature while Factor 2 (y-axis) represents soil amendments (willow root, fertilizer, and salicylic acid). Figure 11 additionally demonstrates that Factor 1 (temperature) explains 96% of the total independent variable influence (x-variable) and 55% of the response variable (DRO loss). Factor 2 (soil amendments) describes 2% of the x-variable and 29% of the response variable. Together both factors describe 98% of the x-variable and 84% of the response variable. The fact that Factor 1 explains close to 100% of the independent variable (x-variable) and Factor 2 explains a very low percentage of the independent variable (x-variable) means that the two factors are independent from each other.

By placing a vector through the correlations loadings graph it can be observed that temperature and crushed fine willow root amendment correlated with DRO loss, as indicated by the angle between the vector and the temperature and crushed fine willow root points.

Salicylic acid on the other hand is located across the axis, indicating a negative correlation between salicylic acid and DRO loss.

Figure 11 also shows fertilizer (beneath the crushed fine willow root point). Although the fertilizer point is within the inner ring (indicating 50% or less of variance explained), the predictive capacity of the PLS model decreased when it was run without fertilizer. This indicates that fertilizer had some influence on diesel loss.

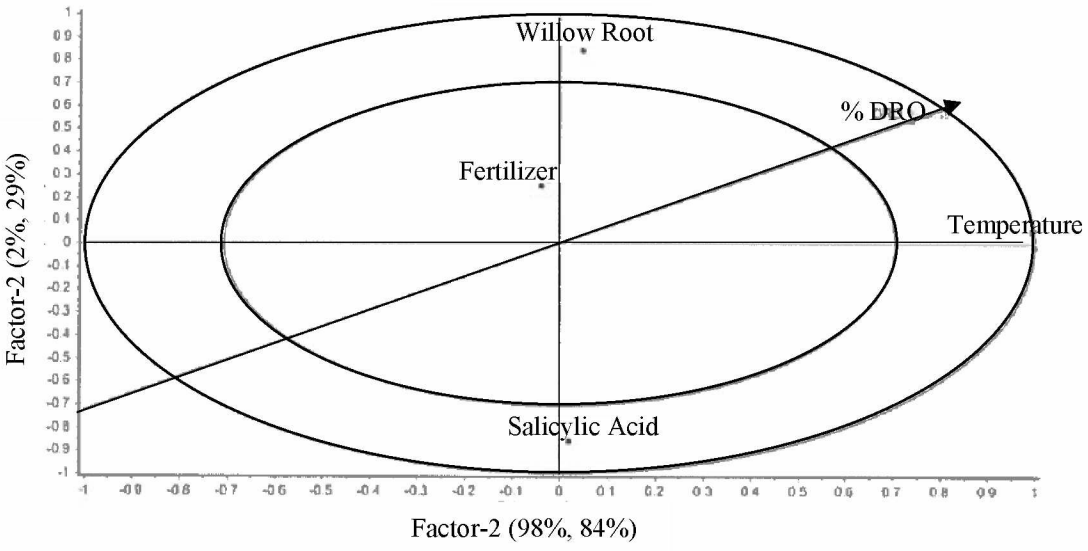

Figure 11: PLS Correlation Loadings for DRO Loss from Weathered-Diesel Microcosms

Results for fresh-diesel spiked microcosms (shown in Appendix F) could not be modeled by PLS, as shown through regression analysis of the referenced and modeled data where the R^2 value was near 0 (data not shown), indicating a weak correlation between the treatment parameters and loss of diesel over 90 days.

3.2 Presence of Phenol

Significant phenol spikes were observed upon analysis of GC/MS chromatograms from various microcosms sampled on Days 14, 45, and 90 (Table 3).

Table 3: Observed Phenol Spikes in GC/MS Spectra

Microcosm #	Temp. (°C)	Soil Type	Parameters	Day 14	Day 45	Day 90
8	20	Contaminated	Fert.	x		
11	20	Contaminated	SA	x	x	x
12	20	Contaminated	SA	x	x	
20	20	Contaminated	SA&Fert.		x	x
32	20	Fresh-Spiked	Fert.	x		
40	20	Fresh-Spiked	WR	x		
87	4	Contaminated	SA		x	x
88	4	Contaminated	SA		x	x
95	4	Contaminated	SA&Fert.		x	x
96	4	Contaminated	SA&Fert.		x	x
111	4	Fresh-Spiked	SA		x	x
112	4	Fresh-Spiked	SA		x	
119	4	Fresh-Spiked	SA&Fert.		x	
120	4	Fresh-Spiked	SA&Fert.		x	
132	4	Clean	SA		x	
133	4	Clean	SA		x	
137	4	Clean	SA&Fert.		x	x

A complete list of microcosm numbers and their associated parameters can be found in Appendix A. Phenol spikes were considered to be significant when they were the largest peak present in the chromatogram.

Spikes in phenol were most commonly associated with the presence of salicylic acid, or salicylic acid and fertilizer, as seen in the table above. The only three exceptions were observed on Day 14. Phenol peaks were omitted from the quantification of total DRO during analysis of GC/MS results. Most phenol spikes were found on Day 45 and almost half of the phenol spikes had dissipated by Day 90. Only microcosm # 11 had a visible phenol spike from Day 14 to Day 90.

3.3 Enumeration of DDM Results

At both 20 ºC and 4 ºC the MPN of DDM in weathered diesel-contaminated soils generally decreased over time, as shown in Figures 12 and 13, which depict the average of duplicate microcosms. At both temperatures the decrease in DDM was most pronounced for microcosms amended with salicylic acid or salicylic acid with fertilizer. At 4 ºC, no culturable DDM were detected in weathered-diesel microcosms amended with salicylic acid and fertilizer on Day 89.

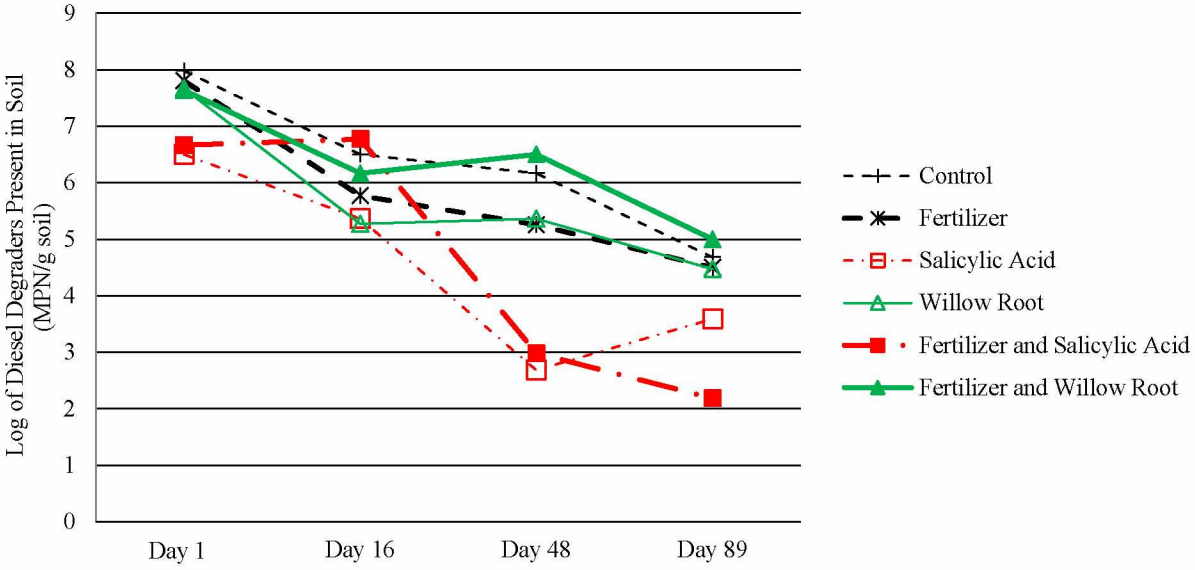

Figure 12: Concentration of DDM in Weathered-Diesel Microcosms at 20 ºC

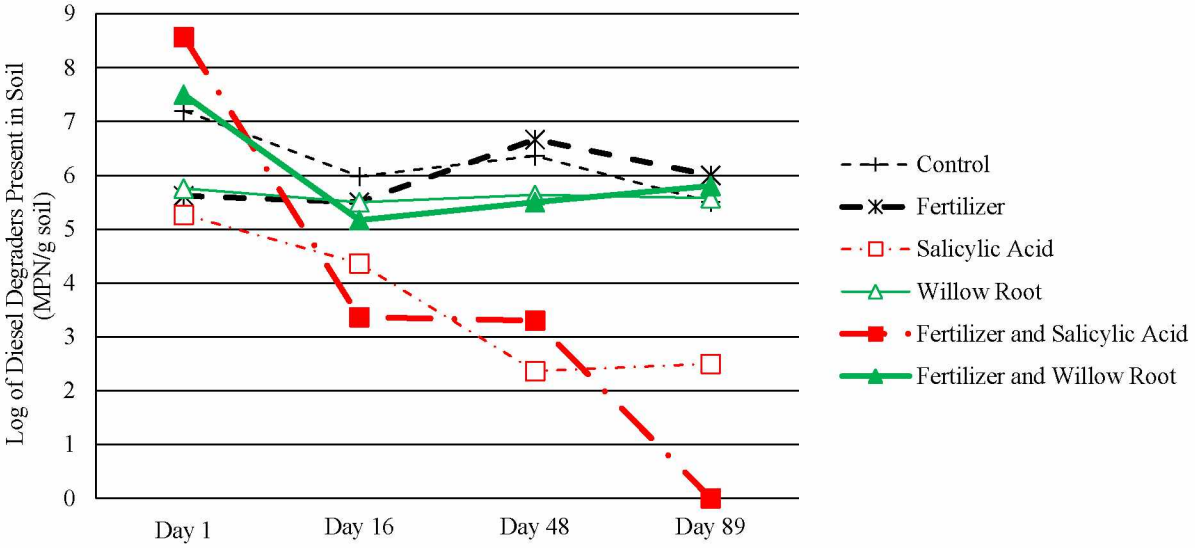

Figure 13: Concentration of DDM in Weathered-Diesel Microcosms at 4 ºC

For microcosms containing fresh-diesel spiked soil, the number of DDM present in the soil generally increased at both temperatures (Figure 14 and Figure 15, showing average of duplicates). At 20 ºC the highest number of DDM was typically present in microcosms amended with crushed willow root and fertilizer (Figure 14). For other soil amendments DDM counts were on average nearly an order of magnitude lower. At 4 ºC microcosms with crushed fine willow root had the highest MPN counts; salicylic acid, as well as salicylic acid and fertilizer, had the lowest MPN counts (Figure 15). On Day 48, no culturable DDM were detected for salicylic acid with fertilizer.

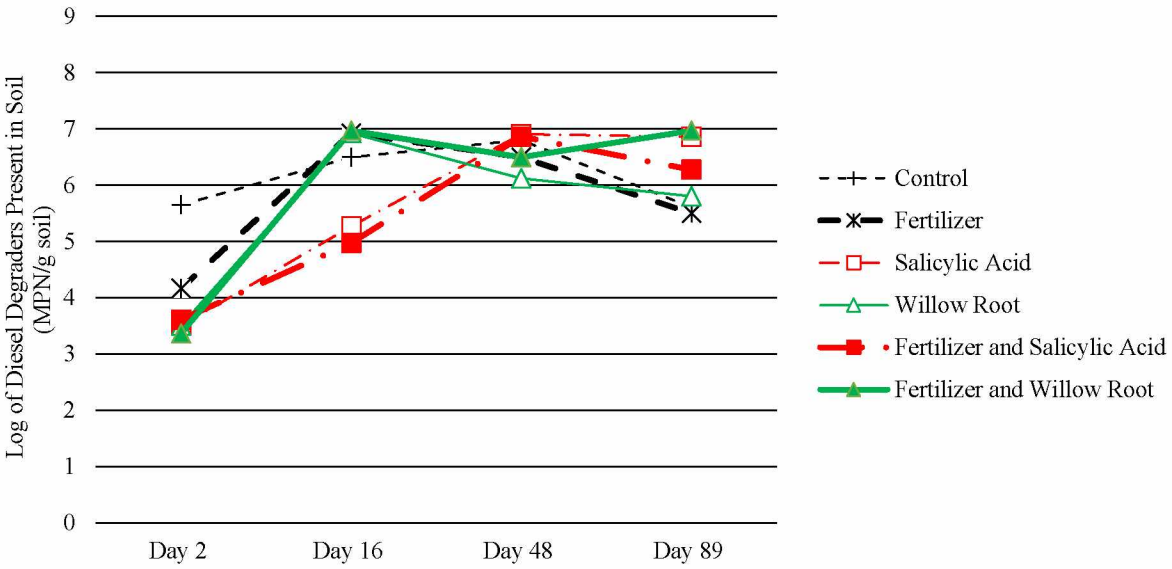

Figure 14: Concentration of DDM in Fresh-Diesel Spiked Microcosms at 20 ºC

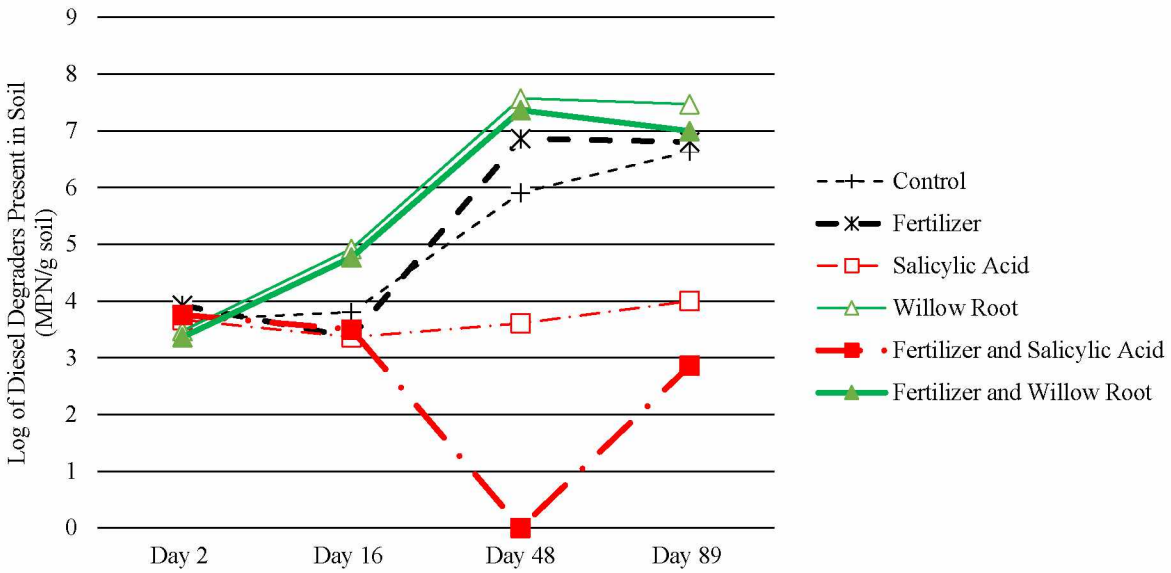

Figure 15: Concentration of DDM in Fresh-Diesel Spiked Microcosms at 4 ºC

The clean soil microcosms showed different patterns of the quantity of DDM over time, depending on temperature. At 20 ºC (Figure 16, showing average of duplicate microcosms), the highest MPN was observed for salicylic acid, followed by salicylic acid and fertilizer (two orders of magnitude higher than the lowest results); the number of culturable DDM increased up to Day 48, and then generally decreased. In contrast, at 4 ºC (Figure 17, showing average of duplicate microcosms) the number of DDM decreased over time, with little difference between the soil treatments, all being within approximately 1-2 orders of magnitude.

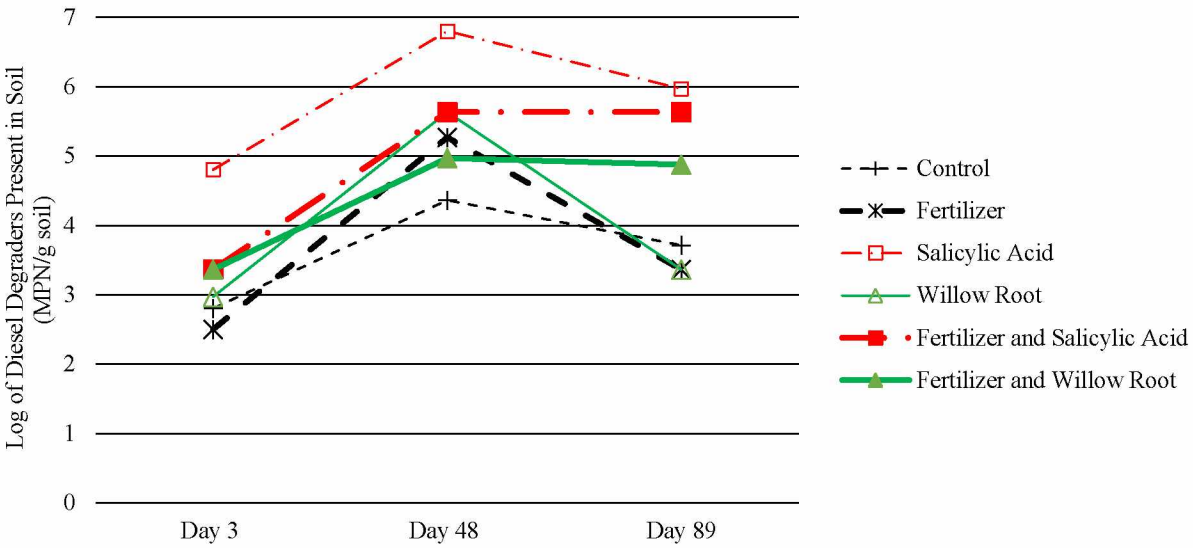

Figure 16: Concentration of DDM in Clean Soil Microcosms at 20 ºC

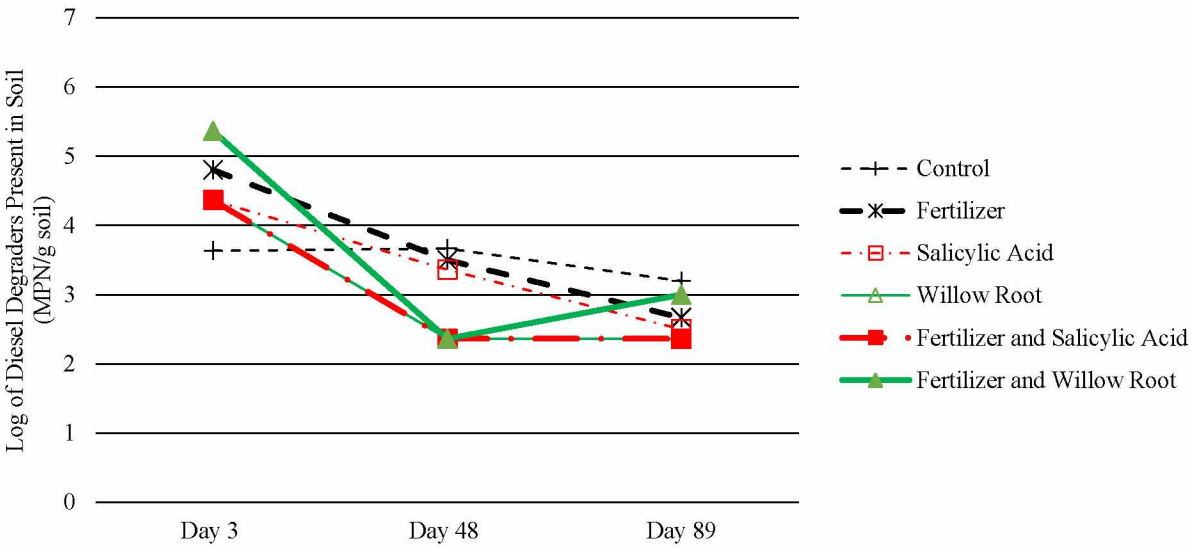

Figure 17: Concentration of DDM in Clean Soil Microcosms at 4 ºC

Microcosms containing fresh diesel-spiked, sterilized soil did not remain sterile under both temperature conditions. Results for the fresh diesel-spiked, sterilized soil can be found in Appendix G.

The PLS regression model did not detect a significant effect from any of the parameters of the study on MPN results (Appendix G).

3.4 Respirometry Results

Weathered diesel microcosms at 20 ºC (Figure 18) showed higher spikes in respiration than at 4 ºC (Figure 19, note different scale). Results in Figure 18 and Figure 19 are the average of duplicated microcosms. In addition, weathered diesel microcosms incubated at 20 °C maintained an overall higher baseline rate when compared to all other microcosm soil types (fresh diesel-spiked and clean). The highest respiration peak (609 g CO_2) at 20 ºC occurred on Day 9 with fertilizer and salicylic acid. For microcosms incubated at 4 ºC, fertilizer amended microcosms showed the highest peak on Day 7, while Day 49 had the highest recorded respiration levels in the control and salicylic acid amended microcosms. Fertilizer was commonly associated with increased respiration at 20 ºC, which was supported by PLS regression as shown below.

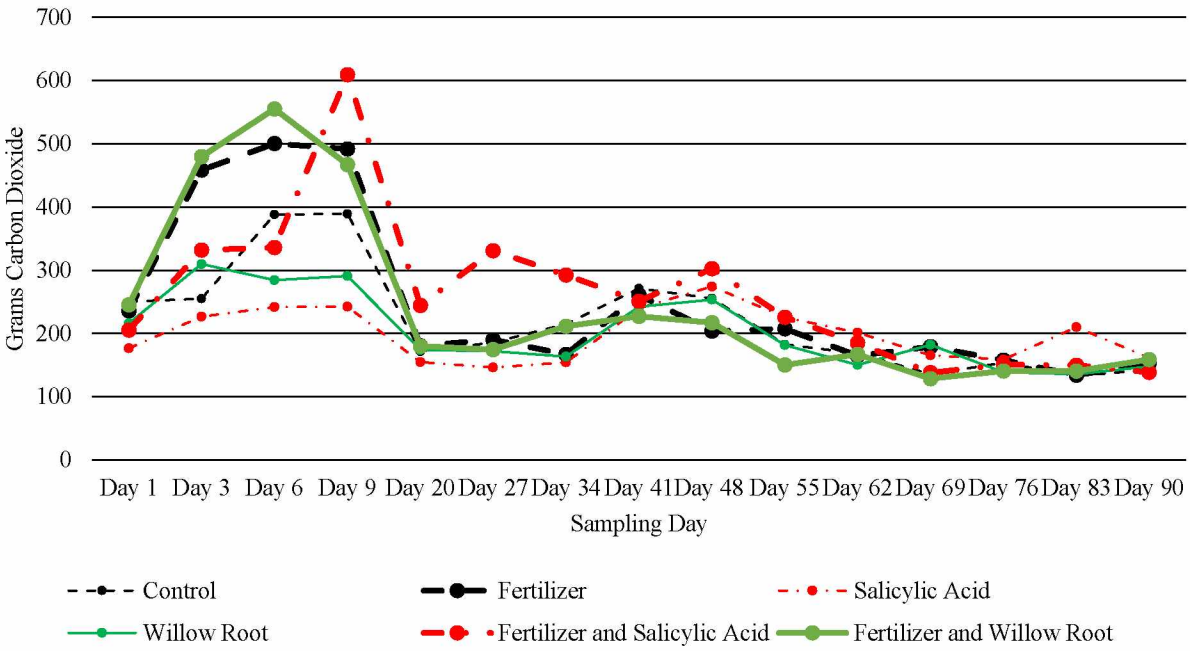

Figure 18: Daily CO_2 Production in Weathered-Diesel Microcosms at 20 °C

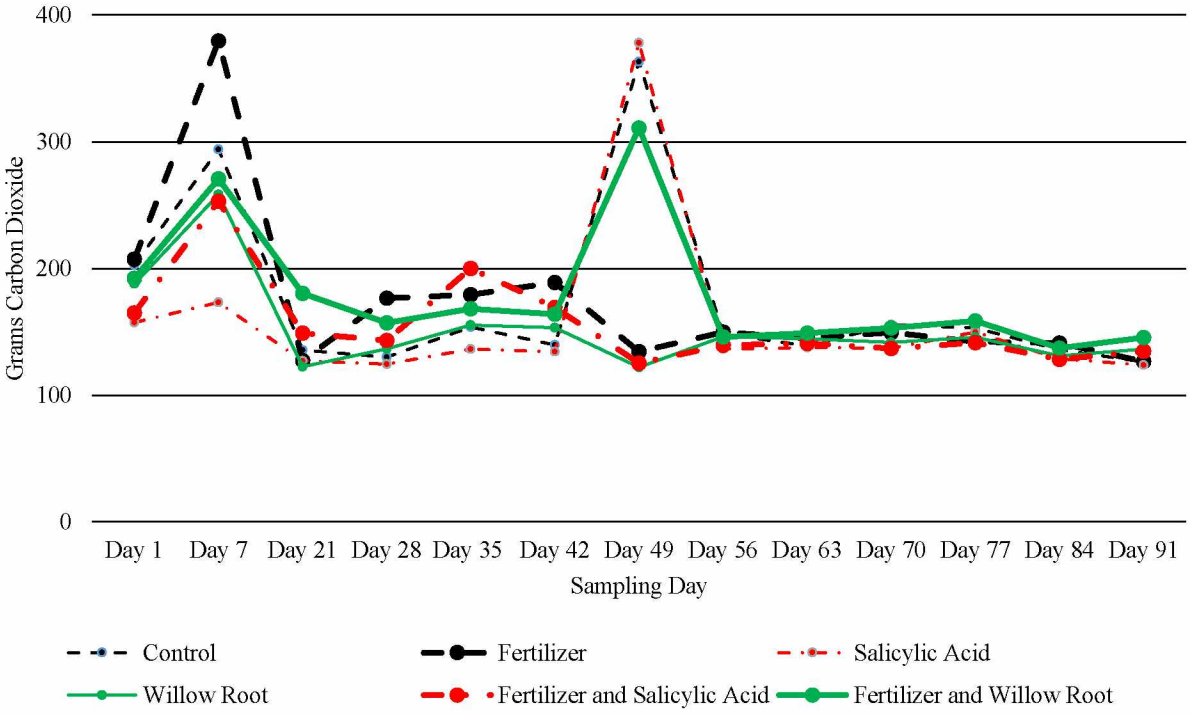

Figure 19: Daily CO_2 Production in Weathered-Diesel Microcosms at 4 °C

PLS regression analysis of respirometry data from weathered-diesel soil microcosms showed that two factors were capable of explaining the data (Figure 20). The calibration and validation explained variances are similar, indicating that the model is reliable. The PLS regression was accurately able to model the data (Figure 21); the R^2 of the calibration data is 0.8775 and R^2 of the model validation data is 0.8233. Factor 1 (temperature) is capable of describing 96% of the x variable and 80% of the response variable, i.e. CO_2 production (Figure 20 and Figure 22).

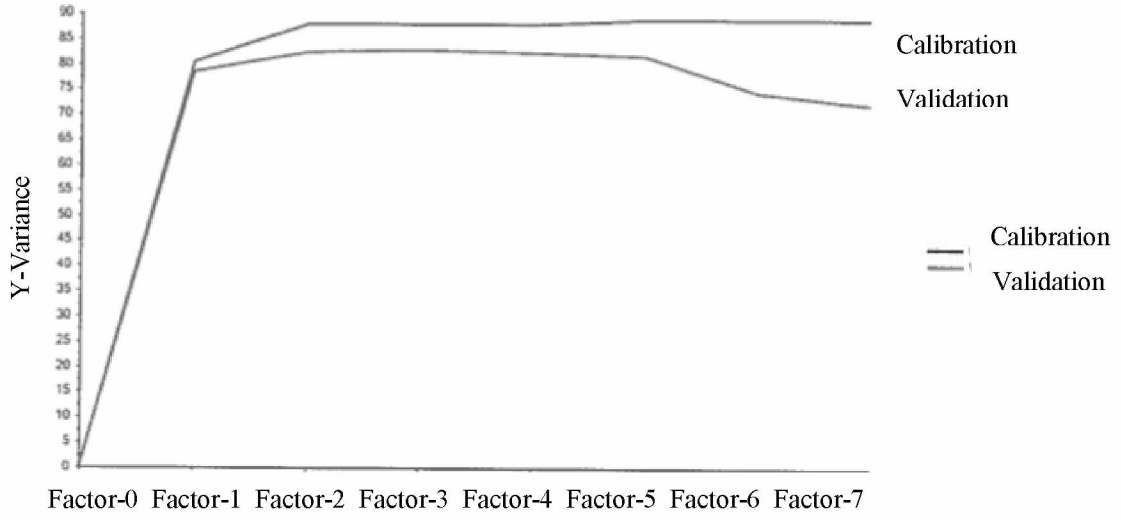

Figure 20: PLS Factors Needed to Explain CO_2 Production from Weathered-Diesel

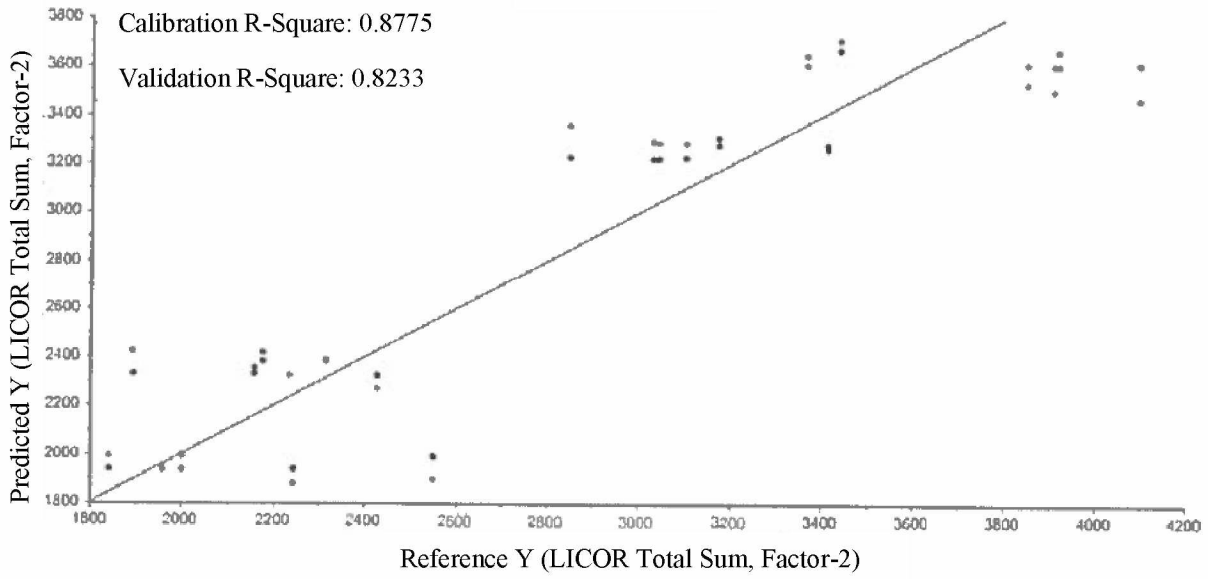

Figure 21: PLS Regression Modeling of CO_2 Production from Weathered-Diesel

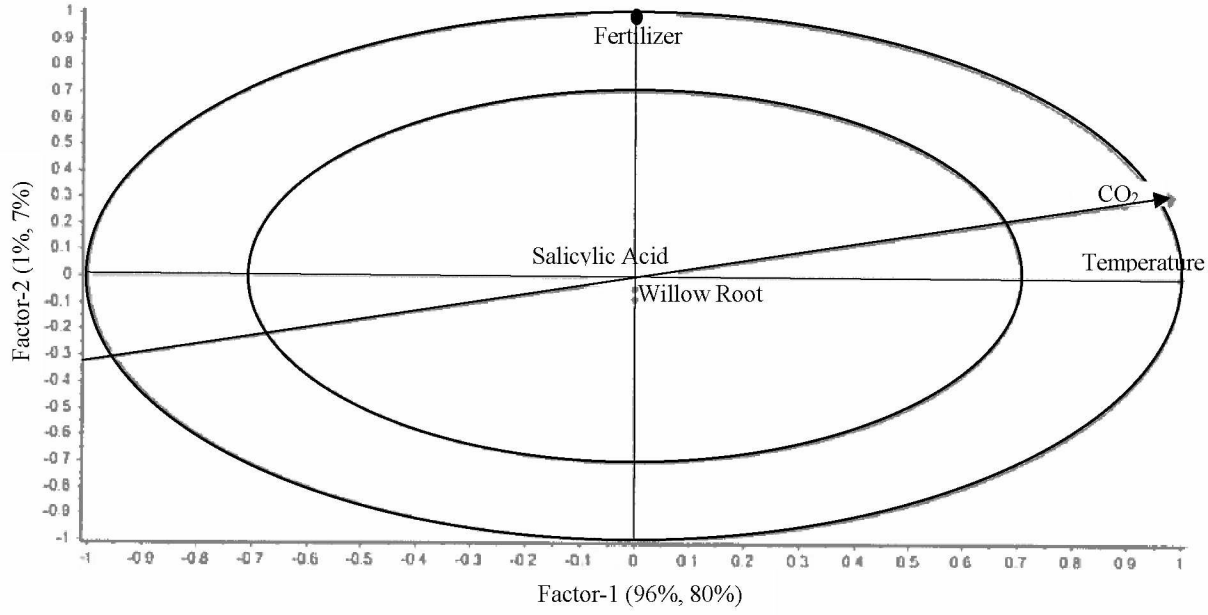

Figure 22: PLS Correlation Loadings for CO_2 Production from Weathered-Diesel

Of the soil amendments, only fertilizer had a positive correlation with the CO_2 production. Willow root and salicylic acid had no effect on respiration as indicated by their location close to the center of the coordinate system. Factor 2 (fertilizer) explained 1% of the x variable and 7% of the response variable, indicating that fertilization only had a minor impact on CO_2 production. Together, Factor 1 and Factor 2 are able to describe 97% of the x variable and 87% of the response variable. After temperature, fertilizer was shown to be the most significant parameter increasing respiration.

Fresh diesel-spiked soil microcosms had higher respiration peaks at 20 ºC (Figure 23) than at 4 ºC (Figure 24), baseline respiration rates were similar at both temperatures (note different y scale). Results in Figure 23 and Figure 24 are the average of duplicate microcosms. Salicylic acid and fertilizer resulted in a peak respiration of 490 g CO_2 for microcosms incubated at 20 ºC on Day 9. At 4 ºC, microcosms with salicylic acid and fertilizer had peak of 235 g CO_2 production at Day 41, the combination of willow root and fertilizer had a peak of 231 g on Day 48.

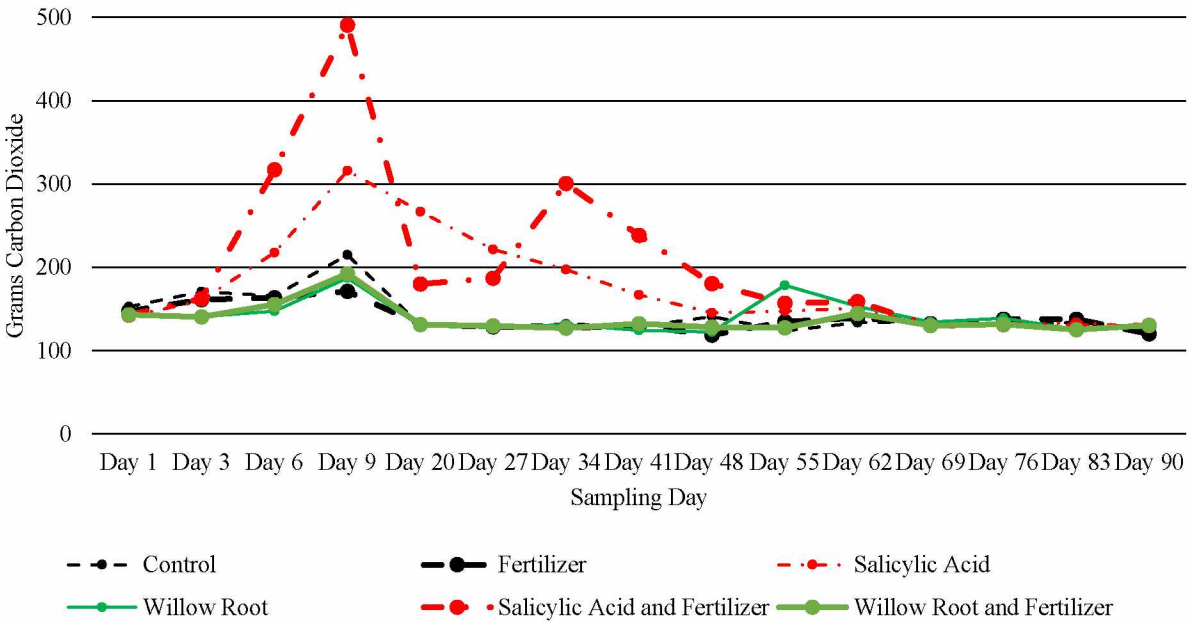

Figure 23: Daily CO_2 Production from Fresh-Diesel Spiked Soil Microcosms at 20 °C

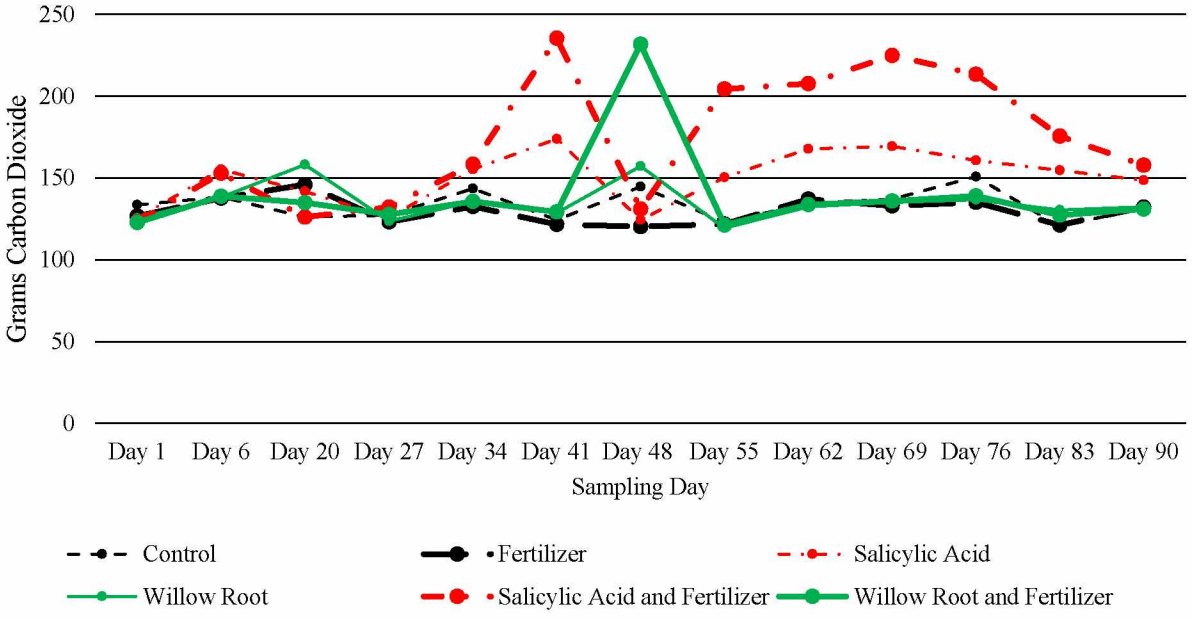

Figure 24: Daily CO_2 Production in Fresh-Diesel Spiked Soil Microcosms at 4 °C

Fresh diesel-spiked, sterilized microcosms at 20 ºC (Figure 25) maintained a steady respiration baseline for all amendments, which was typically between 140 g CO_2 and 120 g CO_2. At 4 ºC (Figure 26) there was a slight peak in respiration to 250 g CO_2 on Day 4 for the control microcosm, the cause of the peak in respiration is unknown. Baseline respiration rates for 4 ºC fresh diesel-contaminated, sterilized microcosms were in a similar range to the 20 ºC incubated microcosms.

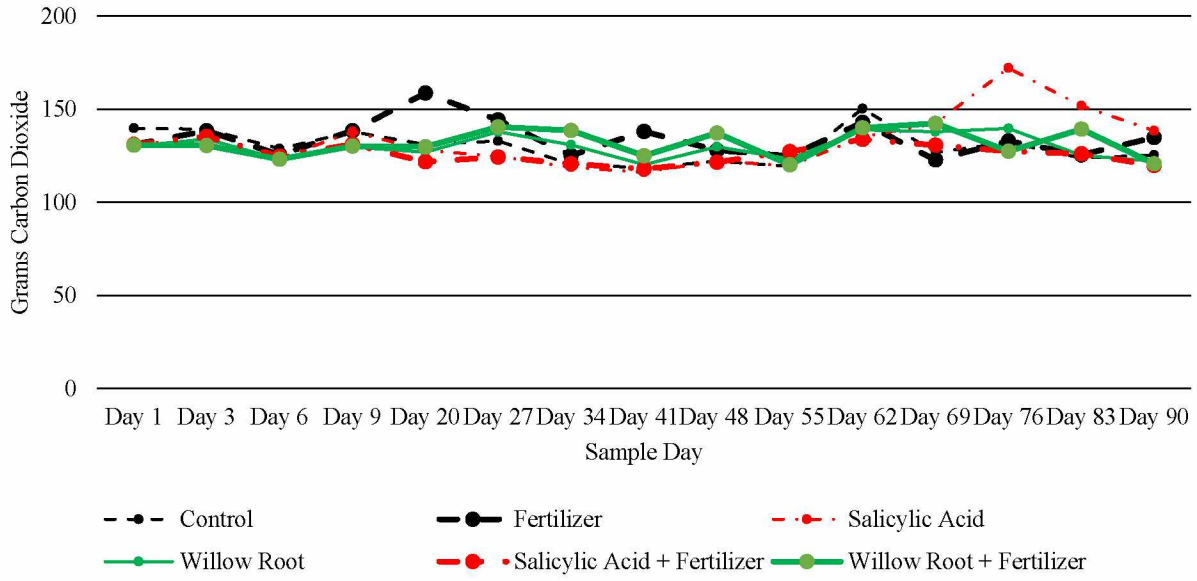

Figure 25: Daily CO_2 Production from Fresh-Diesel Spiked, Sterilized Soil at 20 ºC

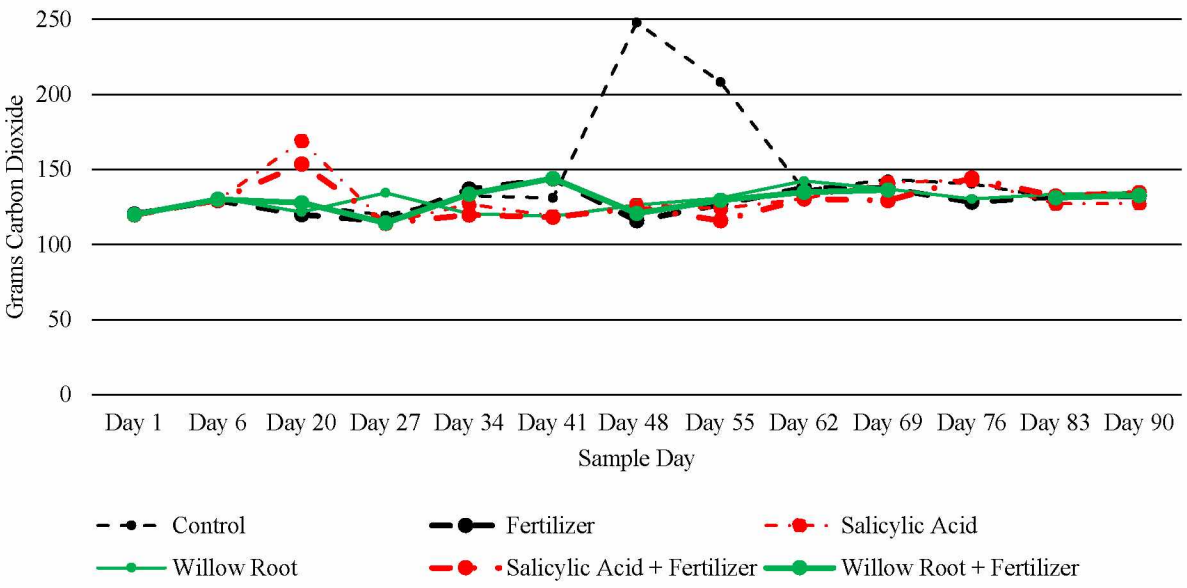

Figure 26: Daily CO_2 Production from Fresh-Diesel Spiked, Sterilized Soil at 4 ºC

PLS regression was performed combining the fresh diesel-spiked microcosms and the fresh diesel-spiked, sterilized microcosm respirometry data, with similar results. Two factors were required to explain the data, as seen in Figure 27, where the explained variance did not improve much when further factors were considered.

For the first 2 factors the model was moderately effective, and this is supported by the regression model seen in Figure 28. The calibration and validation had R^2 values of 0.7022 and 0.6082, respectively, indicating the model was moderately effective utilizing two factors.

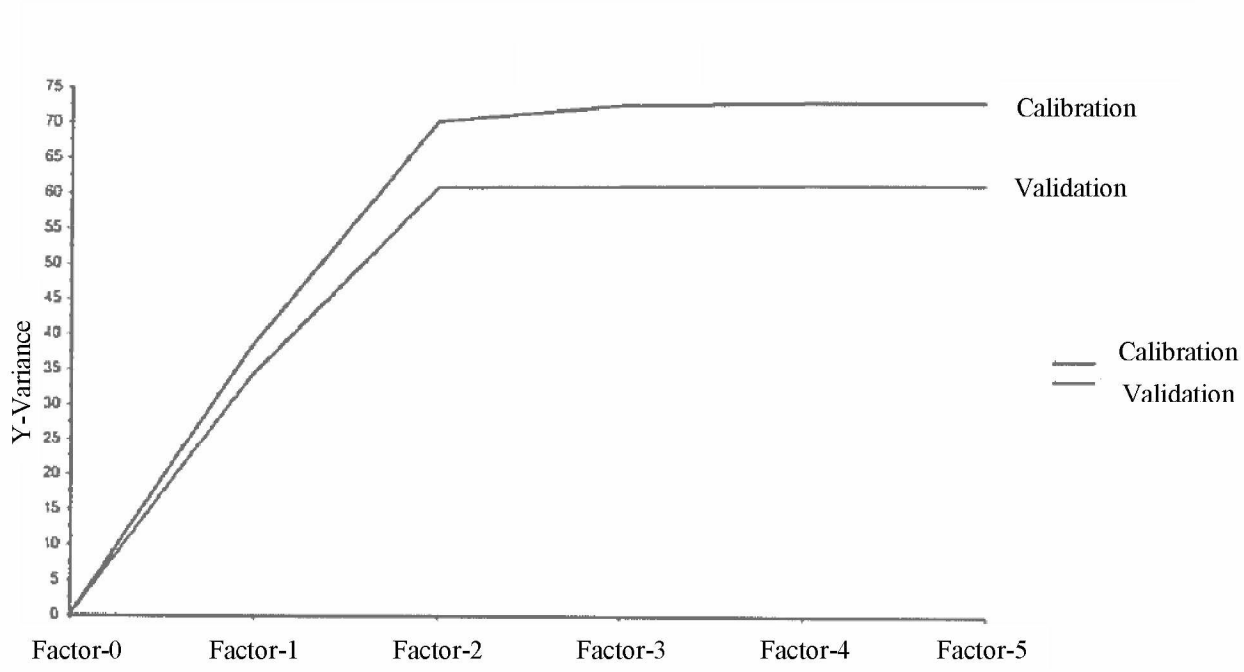

Figure 27: PLS Factors Needed to Explain CO_2 Production from Fresh-Diesel Spiked Soils

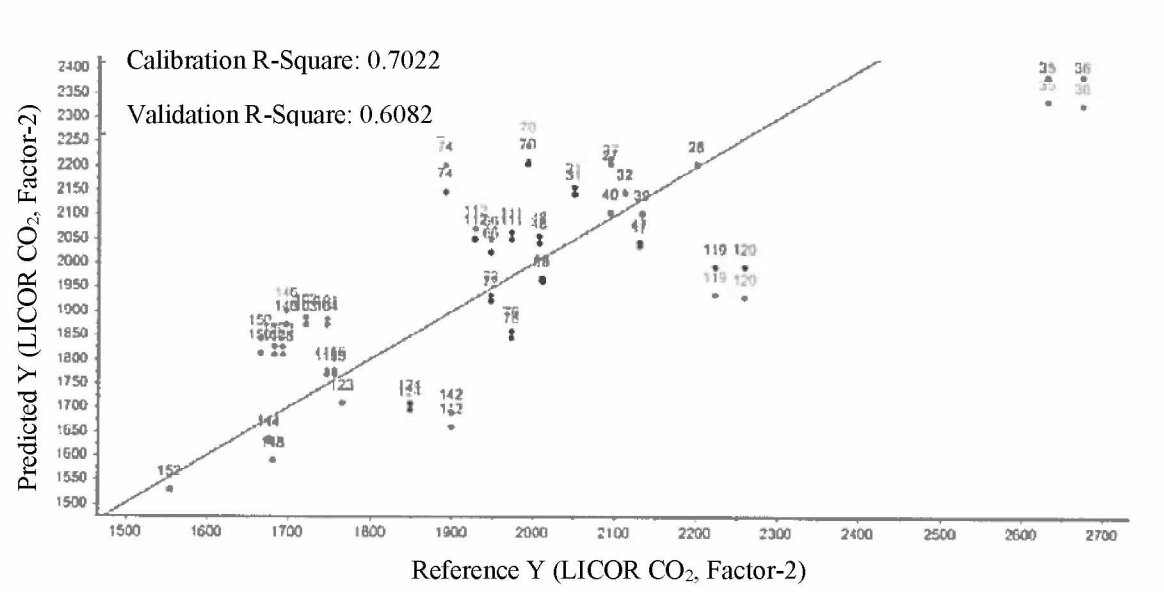

Figure 28: PLS Regression Modeling of CO_2 Production from Fresh-Diesel Spiked Soils

The correlation loading graph in Figure 29 shows the influence of the 2 factors on CO_2 production. Factor 1 (temperature) describes 95% of the x variable and 39% of the response variable. Factor 2 (soil amendments and microbial presence) describes 2% of the x variable and 32% of the response variable. Together these two factors describe 97% of the x variable and 71% of the response variable. Temperature had the strongest influence on CO_2 production, and addition of pure salicylic acid and the absence of sterilization also increased CO_2 production. The resulting influence of salicylic acid on CO_2 production is the result of the average sample data shown in Figure 23 and Figure 24.

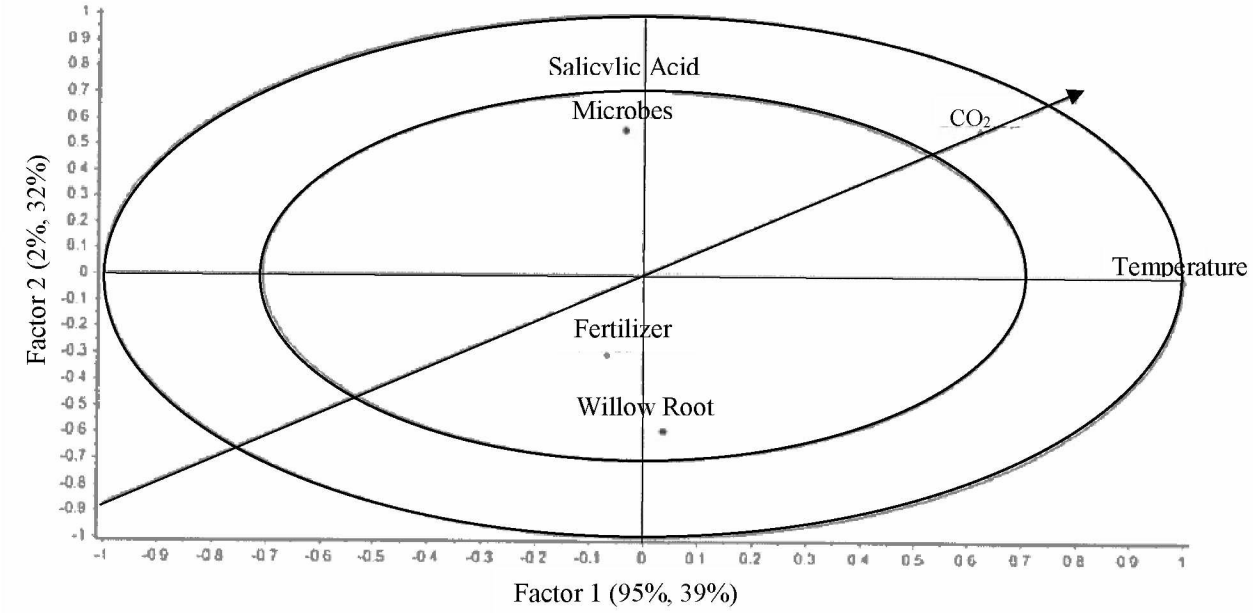

Figure 29: PLS Correlation Loadings for CO_2 Production from Fresh-Diesel Spiked Soils

The PLS regression modeling of respiration in clean soil microcosms can be found in Appendix H.

3.5 Observed Fungal Growth

During the 90 day experiment, fungi started to grow in multiple LI-COR microcosms (Figure 30). Microcosms that had visible fungal growth are shown in Table 4, and a complete list of microcosm jars and associated parameters is found in Appendix A.

Figure 30: Fungal Growth Observed in Microcosm # 34

Table 4: Observed Fungal Growth

				Day Present									
Microcosm Number	Soil Type	Parameter	Temp. (°C)	9	13	20	27	34	35	41	48	55	62
17	Weathered	SA&Fert.	20				x	x					
18	Weathered	SA&Fert.	20				x						
23	Weathered	WR&Fert.	20		x	x							
33	Fresh-Spiked	SA	20	x	x	x	x	x	x	x	x	x	x
34	Fresh-Spiked	SA	20	x	x	x	x	x	x	x	x	x	x
41	Fresh-Spiked	SA&Fert.	20	x	x	x	x	x	x	x	x	x	x
42	Fresh-Spiked	SA&Fert.	20	x	x	x	x	x	x	x	x	x	x
45	Fresh-Spiked	WR&Fert.	20								x	x	
55	Clean	SA	20	x	x	x	x						
60	Clean	SA&Fert.	20	x	x	x					x	x	
61	Clean	SA&Fert.	20							x			
66	Spiked Sterile	Control	20					x	x	x			
67	Spiked Sterile	Fert.	20				x	x					x
68	Spiked Sterile	Fert.	20				x	x					
70	Spiked Sterile	SA	20							x			
71	Spiked Sterile	WR	20				x	x	x	x	x	x	x
72	Spiked Sterile	WR	20				x	x	x	x			
75	Spiked Sterile	WR&Fert.	20				x	x	x	x	x	x	x
136	Clean	SA&Fert.	4						x				
137	Clean	SA&Fert.	4						x				

Almost all of the fungal growth was observed in microcosms incubated at 20 ºC. The only 4 ºC microcosms with fungal growth contained clean soil amended with fertilizer and salicylic acid (Microcosms # 136 and 137). Sustained fungal growth on all days was only observed in those microcosms containing fresh diesel-spiked soil amended with salicylic acid or salicylic acid and fertilizer. One fresh diesel-spiked microcosm that contained crushed fine willow root and fertilizer (Microcosm # 45) showed fungal growth on Day 48 and Day 55.

Weathered diesel-contaminated soil microcosms incubated at 20 ºC showed fungal growth in soils amended with salicylic acid and fertilizer or crushed fine willow root and fertilizer.

Microcosms containing clean soil did have fungal growth at 20 ºC for those amended with salicylic acid or salicylic acid and fertilizer. Clean soil microcosms with salicylic acid and fertilizer were the only ones that also exhibited fungal growth at 4 ºC.

Fungal growth was observed in fresh diesel spiked, sterilized soil microcosms incubated at 20 ºC from day 27 onward, which appears to be due to spores being introduced before that day.

3.6 Microtox® Analysis Results

Microtox results were recorded as EC50 (mg/L) i.e. the effective concentration that resulted in a 50% decrease in the light emitted by the *V. fischeri*. Using this scale, increased toxicity is associated with lower EC50 values. Microtox results were collected from weathered-diesel microcosms incubated at 20 °C and 4 °C on Day 1 (Figure 31, showing average of duplicate microcosms), and from 20 °C microcosms on Day 90. Figure 31 shows that on Day 1 of the study, weathered diesel-contaminated soils incubated at 20 °C were typically less toxic (higher EC50) than those incubated at 4 °C on Day 1 of the study. The addition of salicylic acid did influence soil toxicity, soil amended with salicylic acid had a lower EC50. At 20 °C, the

weathered diesel-contaminated control (no soil amendments added) was the least toxic while any soil amendments, especially salicylic acid and fertilizer, resulted in greater toxicity at 20 ℃ and 4 ℃.

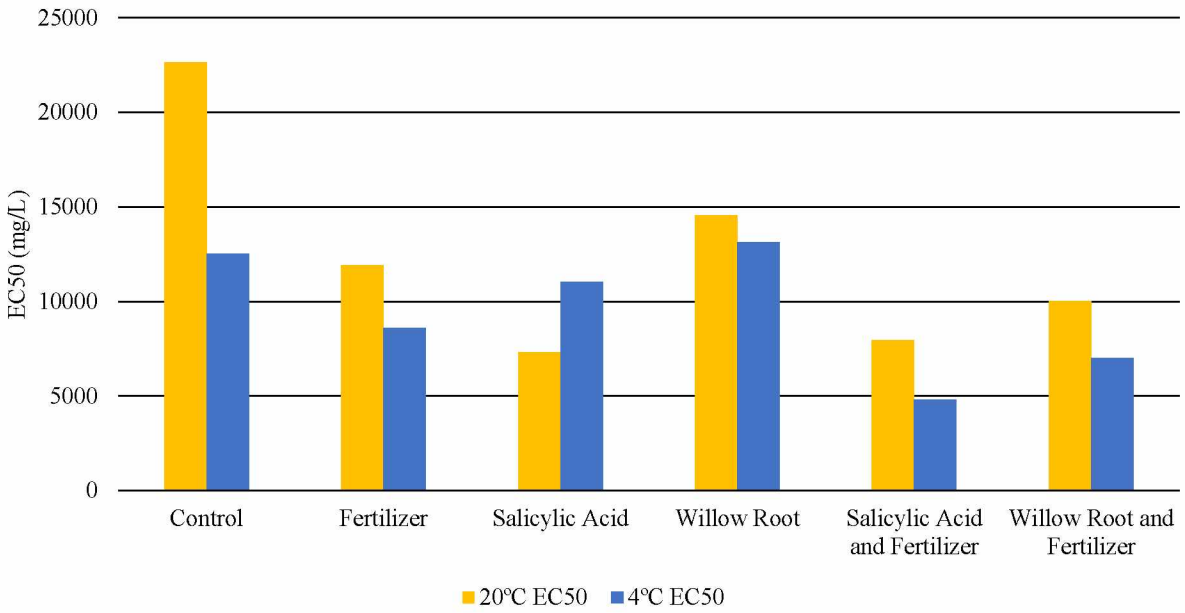

Figure 31: Microtox® Results in Weathered-Diesel Microcosms on Day 1

The PLS regression model was able to moderately model the EC50 results for the weathered diesel-contaminated microcosms at both 20 ℃ and 4 ℃ on Day 1 utilizing 2 factors (Figure 32). The calibration and validation explained variance are relatively separated in distance, indicating that the model is only somewhat effective for predicting toxicity. This is supported by the regression graph, shown in Figure 33, with R^2 values of 0.8028 and 0.7023 for the calibration and the validation respectively.

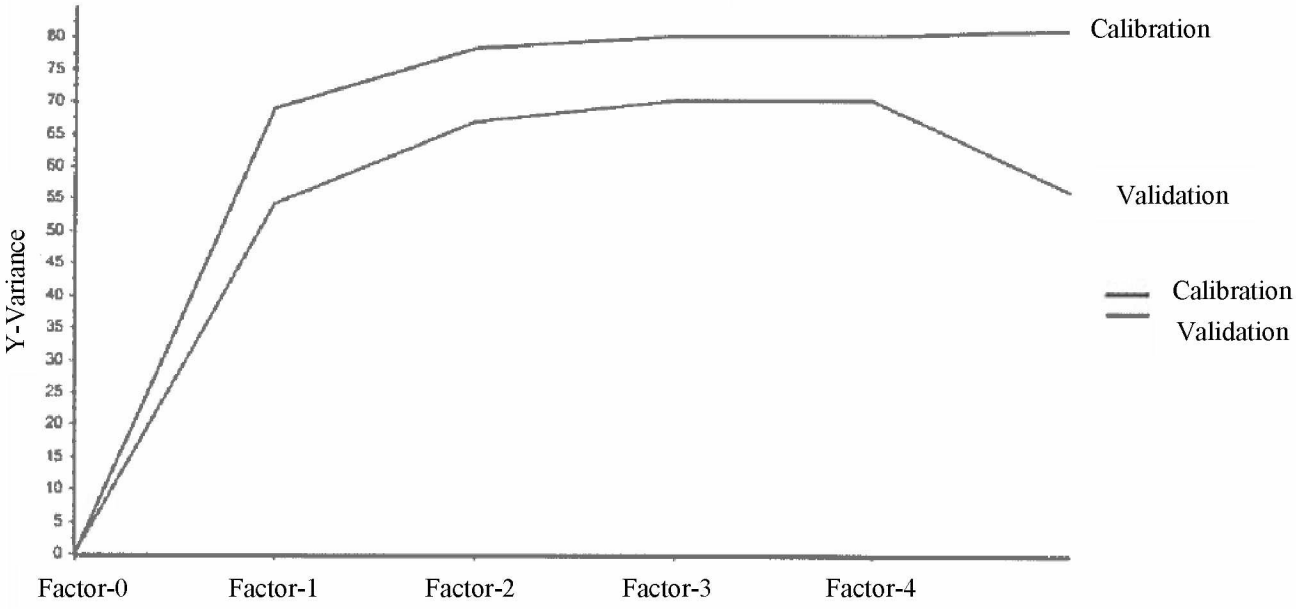

Figure 32: PLS Factors Required to Explain Microtox® for Weathered-Diesel Microcosms

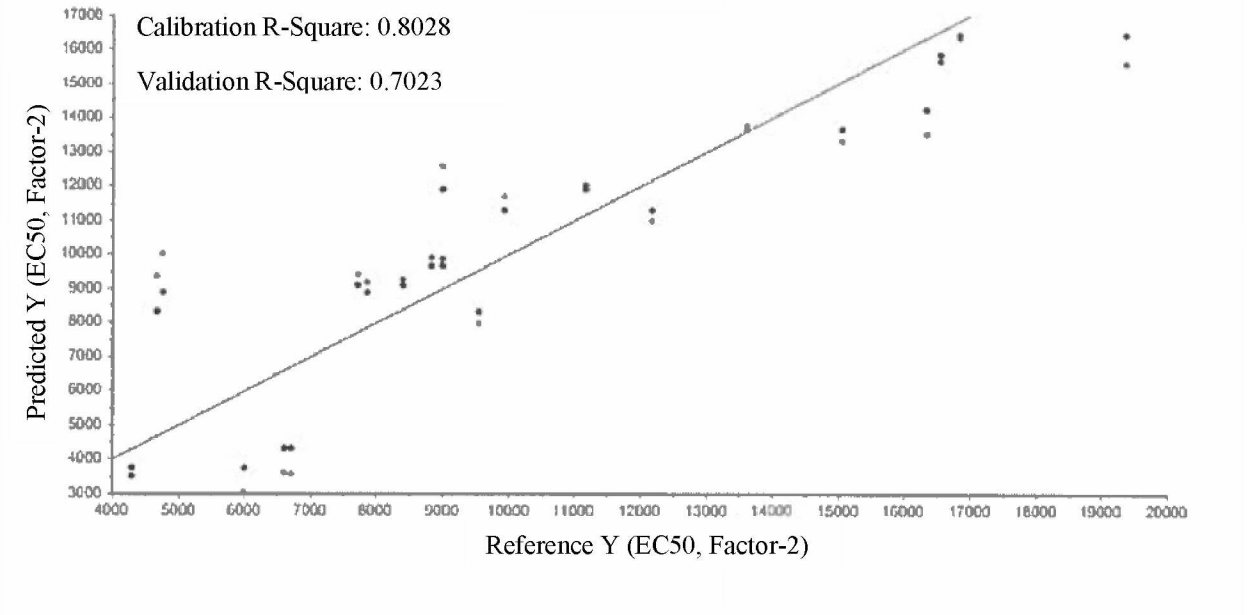

Figure 33: PLS Regression Modeling of Microtox® for Weathered-Diesel Microcosms

In the correlation loadings plot (Figure 34), factor 1 is mainly based on the soil amendments salicylic acid and fertilizer and explains 29% of the independent variable influence (x-variable) and 69% of the response variable (Toxicity-EC50). Factor 2 is mainly based on willow root (with some effect of fertilizer) and explains 24% of the independent variable (x-variable) and 9% of the response variable (EC50). Based on the PLS results, salicylic acid and fertilizer most strongly influenced the increase in toxicity, visualized by the vector in Figure 34.

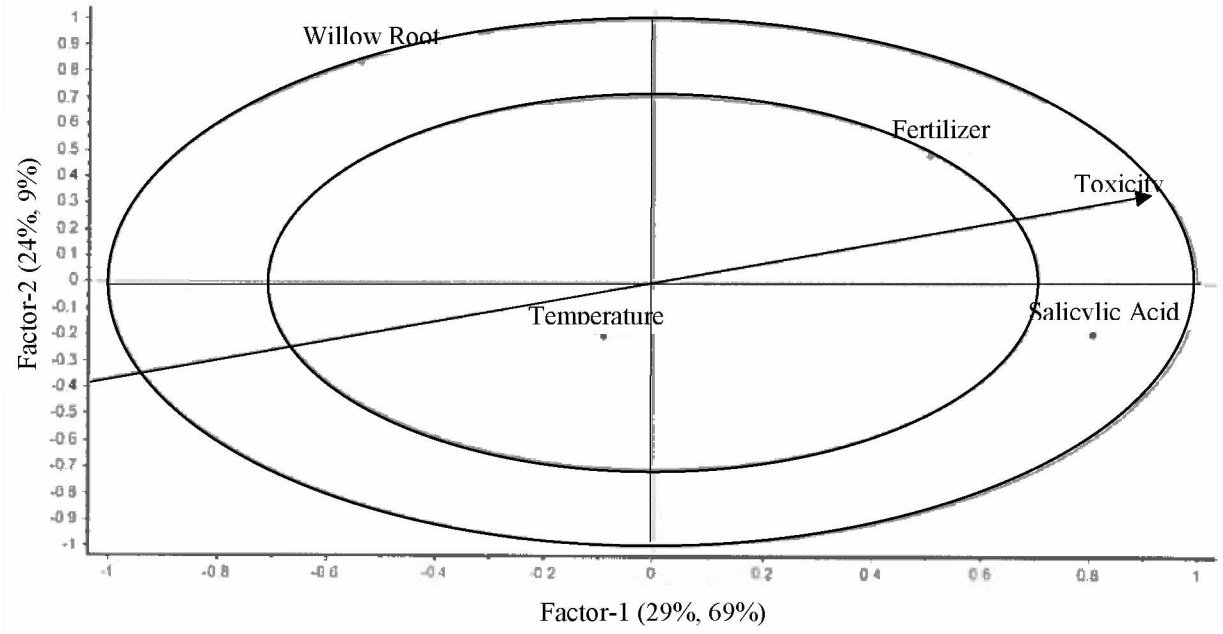

Figure 34: PLS Correlation Loadings of Microtox® for Weathered-Diesel Microcosms

EC50 results for the toxicity of weathered diesel soil, incubated at 20 ºC, from Day 1 to Day 90 showed a trend of increasing toxicity over the course of the experiment (Figure 35). The greatest increase in toxicity over the course of the study was for the weathered diesel control microcosms, which initially had the lowest toxicity and finally the highest one. The only case where EC50 increased over the 90 day study was for weathered diesel soils amended with crushed fine willow root and fertilizer; these microcosms showed a decrease in toxicity. At the end of the 90 day study microcosms amended with crushed fine willow root had the highest EC50. Of the soil amendments salicylic acid microcosms showed to be the most toxic.

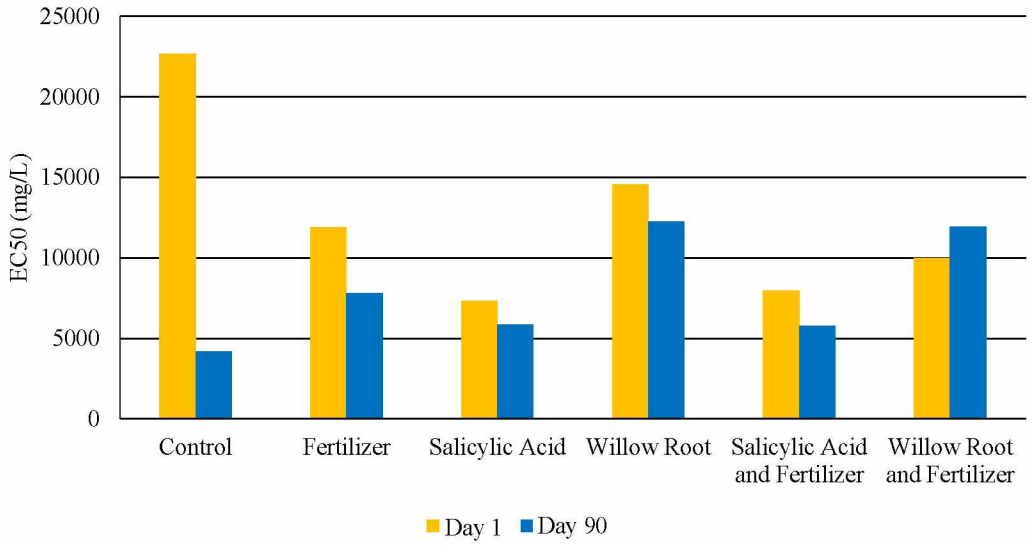

Figure 35: Microtox® Results for Weathered-Diesel Microcosms at 20 ºC

Section 4 Discussion and Conclusion

The overarching objective of our study was to determine, based on microcosm studies, if *S. alaxensis* should be recommended for a field trial of phytoremediation for diesel contaminated soil in the community of Kaltag, Alaska. We hypothesized that the addition of salicylic acid or crushed fine willow roots (simulating fine root turnover of *S. alaxensis*) would increase diesel loss from microcosms. To test our hypothesis, a microcosm study was conducted to test the impacts of temperature, crushed fine willow root, salicylic acid and nutrient addition on fresh or weathered diesel loss in soil from Kaltag, Alaska.

As expected, weathered diesel loss was greater at 20 ºC as a result of increased microbial activity and volatilization. This was supported by GC/MS analysis and PLS analysis of sample results. The increase in weathered diesel loss from soils incubated at 20 ºC is in line with Yang et al. (2009) and Walworth et al. (2001) who found diesel degradation from sub-arctic soils was higher during summer months (15-20 ºC). The increased weathered diesel loss from soils incubated at 20 ºC, shown during GC/MS analysis, did not coincide with an increase in culturable diesel degraders from MPN testing. The observed weathered diesel loss may have been the result of an increase in non-culturable diesel degraders and/or an increase in cellular activity, cellular activity is increased under warmer temperatures. In addition, warmer temperatures increase the ability of diesel to cross the cell membrane allowing for the diesel to be available for breakdown by DDM. CO_2 production from weathered diesel microcosms was increased during the beginning of the study, and an overall higher baseline respiration rate was maintained for the 90 days when compared to other soil types studied. The levels of CO_2 indicate increased microbial activity, during increased microbial activity there is greater cellular respiration. Respiration was anticipated to be at its greatest during the beginning of the study as

microorganisms would be taking advantage of the easier to degrade components. After the initial peak in respiration, a steady baseline rate was achieved for the remainder of the study. The baseline respiration of the 20 ºC incubated microcosms was elevated when compared to other soil types incubated at 20 ºC.

Although weathered diesel loss was decreased during incubation at 4 ºC, GC/MS analysis showed that weathered diesel loss still occurred. Kaltag, Alaska is within the sub-Arctic and much of year is dominated by winter temperatures, it is important that remediation be able to occur under those conditions. The observed diesel loss was most likely the result of DDM activity. MPN analysis showed that weathered diesel soils incubated at 4 ºC did have culturable DDM present and the number of culturable DDM was similar to those observed in weathered diesel soils incubated at 20 ºC. Even though numbers of culturable DDM were similar between the 20 ºC and 4 ºC incubated soils, diesel loss was lower at 4 ºC as a result of decreased microbial activity and limited availability of diesel. Based on analysis of CO_2 production, microbial activity was greatest at the beginning of the study when easier to degrader components were available. Following the increase in respiration CO_2 production reached a steady rate, this baseline respiration rate was only slightly less when compared to the baseline respiration rate of weathered diesel microcosms incubated at 20 ºC. The baseline respiration rate of 4 ºC weathered diesel soils was also higher than the baseline respiration rates of other soil types incubated at 4 ºC.

Addition of crushed fine willow roots (simulating fine root turnover), in combination with nutrient addition, did enhance weathered diesel loss in microcosms at 20 ºC and 4 ºC as determined by GC/MS analysis and PLS analysis. Separate the addition of nutrients and crushed fine willow root allowed for slight increases in weathered diesel loss, and together they resulted

in the greatest amount of weathered diesel loss. The greater loss could have been caused by a two-fold effect of the nutrient addition and crushed fine willow root. The weathered diesel soils nutrient content was poor prior to the start of the experiment, determined by liquid chromatography, and the addition of nutrients should have resulted in greater cellular activity. Simulated fine root turnover should have also resulted in released nutrients and secondary plant compounds, secondary plant compounds have the capability to induce gene expression necessary for contaminant degradation. It could not be determined if salicylic acid was the secondary plant compound influencing diesel loss. The influence of soil amendments on microbial activity could not be determined through LI-COR CO_2 production analysis, based on PLS analysis.

Salicylic acid could not be confirmed as the compound from fine root turnover which increased weathered diesel loss, to the contrary, PLS analysis showed a negative correlation between weathered diesel loss and addition of salicylic acid. Weathered diesel microcosms that were amended with pure salicylic acid showed a decrease in culturable DDM, based on MPN testing and PLS analysis. Most likely the concentration of pure salicylic acid was toxic to culturable DDM. The addition of pure salicylic was found in studies by Chen and Aitken (1999) to inhibit microbial activity at concentrations higher than 0.3 mM. By comparison for our study 0.615 M of salicylic acid was utilized. GC/MS analysis revealed that microcosms which contained salicylic acid showed increases in phenol over the course of the experiment. The phenol may have been an intermediate product of salicylic acid breakdown. It was not determined if the presence of salicylic acid itself or the production of phenol influenced the decrease in DDM and/or increased toxicity. Toxicity and reduced DDM numbers can, however, explain the reduced diesel loss from microcosms with salicylic acid as being due to reduced microbial activity.

Microtox® results indicated that on Day 1 of the study, in general, weathered diesel microcosms incubated at 20 °C were less toxic than soils incubated at 4 °C. This may have been because temperature influenced the concentration diesel components. On Day 1 of the study the addition of soil amendments to microcosms incubated at 20 °C and 4 °C always resulted in an increase in toxicity, of the amendments salicylic acid and fertilizer in combination resulted in the greatest increase of toxicity. The greater increase in toxicity as a result of the salicylic acid and fertilizer was supported by PLS analysis. This may have been the result of a two-fold effect. Of the soil amendments the addition of crushed fine willow root to weathered diesel microcosms resulted in the smallest increase of toxicity.

Weathered diesel microcosms incubated at 20 °C had Microtox® samples collected on Day 1, as well as Day 90 of the study. Although the results could not be described by PLS analysis there are possible trends worth noting. Over the course of the 90 day study the control microcosm became the most toxic, even more toxic than amended microcosms. It is possible that the remaining concentration of weathered diesel compounds influenced the results. Of the soil amendments salicylic acid remained the most toxic. Even though the control was more toxic than salicylic acid, salicylic acid had the lowest losses of diesel over the 90 day study. Resulting from the negative impact of the salicylic concentration on DDM, the presence of phenol, or a combination of both. The Microtox® test is an acute toxicity test and although the salicylic acid amended soil was not as acutely toxic on Day 90 to the *V. fisheri* as the control, the toxicity of the salicylic acid was captured by the Microtox® and it is possible that during a chronic toxicity study the salicylic may prove to be the more toxic. It was shown that over the 90 day study the presence of DDM was decreased over the course of the study in weathered diesel microcosms amended with salicylic acid. The only amendment to show a decrease in toxicity over the course

of the study was weathered diesel microcosms containing crushed fine willow root and fertilizer in combination. The same amendments were found to have resulted in the greatest weathered diesel loss.

Fresh diesel loss could not be accurately determined by GC/MS analysis, most likely as the result of volatilization during microcosm setup.

Microcosms that contained sterilized soil showed presence of DDM over the course of the experiment, indicating that the soil was not successfully sterilized.

To test our hypothesis and determine if the other arching objective of our study was met we had a series of questions that we wanted to answer.

- We asked if DDM were present in soils from Kaltag, Alaska and can those present assist in rhizoremediation, even at environmentally relevant temperatures? Over the course of our study we have determined that weathered diesel soil from Kaltag, Alaska can bioremediate on its own as DDM are present at summer and winter temperatures (20 °C and 4 °C). DDM are present in soil from Kaltag, Alaska even when there is no diesel present in the soil and summer and winter temperatures.
- What is the intrinsic diesel degradation of the weathered-diesel and fresh-diesel contaminated soil from Kaltag, AK? An intrinsic diesel degradation rate could not be determined, but our study did show that without the addition of soil amendments that weathered-diesel degradation did occur.
- Is the weathered-diesel contaminated soil from Kaltag, AK toxic to microorganisms? We determined that even in the presence of the weathered-diesel soils still contained viable DDM.

- Does the addition of crushed fine willow roots, salicylic acid and/or fertilizer increase diesel loss and CO_2 production? The presence of crushed fine willow root (used to simulate fine root turnover) and fertilizer results in higher weathered diesel loss over the course of a typical Alaskan growing season (90 days) as well as during summer and winter conditions (4 °C).
- Will intrinsic biodegradation or simulated rhizoremediation reduce the toxicity of diesel-contaminated soil? The toxicity studies performed were difficult to draw conclusions from. The addition of crushed fine willow root and fertilizer did show a decrease in toxicity over the course of the 90 day study. It is likely that the influence of crushed fine willow root and fertilizer on the increased loss of weathered diesel and the presence of secondary plant compounds from the simulated fine root turnover of *S. alaxensis* which may also assist in reducing the toxicity of the soil.

The overarching objective of our study was to determine, based on microcosm studies, if *S. alaxensis* should be recommended for a field trial of phytoremediation for diesel contaminated soil in the community of Kaltag, Alaska. As a result of our study and the answers obtained, we recommend that *S. alaxensis* be recommended for a field trial in Kaltag, Alaska. A diesel contaminated soil potted *S. alaxensis* study performed by Kelly McFarlin (2009) furthers our recommendation.

Section 5 Future Research

5.1 Recommendations for Future Research

Our study showed that additional work is required to understand the role of salicylic acid as well as other secondary plant metabolites in rhizoremediation. This requires identifying which plant metabolites can assist or hinder diesel remediation and which plants are able to produce those metabolites, such as phenol. *S. alaxensis* is just one willow species present in the sub-Arctic, other willows or other plant species may allow for more effective rhizoremediation. Possibly, plant species can be used in combination with one another. This work should not only be performed in the laboratory, but also through field studies. Given the unique weather conditions found throughout Alaska, the laboratory setting cannot always accurately mimic field conditions. By furthering research into the role of plants and secondary root compounds in rhizoremediation, plants may become a common remediation method implemented by Alaska regulators.

5.2 Kaltag Phytoremediation Field Study

After the initiation of our research, contractors excavated an estimated 1,606 cubic meters of contaminated soil during the summer of 2014. Soil was transported to a pre-selected area where it was divided into two plots. One plot is landfarmed by SWI, while the second plot is operated by UAF for phytoremediation studies. The phytoremediation study performed by UAF will be compared against the conventional landfarm method. The UAF field study will compare *S. alaxensis* and *Arctagrostis latifolia* (Alyeska polargrass) in combination and separate, as well as the presence and absence of nutrients, and control plots.

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Appendix A

Microcosms Reference Lists

Table 5: List of Weathered-Diesel Microcosms

20 °C Contaminated Kaltag Soil

Microcosm Number	Amendments
1	Control
2	Control
3	Control
4	Control
5	Fertilizer
6	Fertilizer
7	Fertilizer
8	Fertilizer
9	Salicylic Acid
10	Salicylic Acid
11	Salicylic Acid
12	Salicylic Acid
13	Crushed Fine Willow Root
14	Crushed Fine Willow Root
15	Crushed Fine Willow Root
16	Crushed Fine Willow Root
17	Salicylic Acid and Fertilizer
18	Salicylic Acid and Fertilizer
19	Salicylic Acid and Fertilizer
20	Salicylic Acid and Fertilizer
21	Crushed Fine Willow Root and Fertilizer
22	Crushed Fine Willow Root and Fertilizer
23	Crushed Fine Willow Root and Fertilizer
24	Crushed Fine Willow Root and Fertilizer

4 °C Contaminated Kaltag Soil

Microcosm Number	Amendments
77	Control
78	Control
79	Control
80	Control
81	Fertilizer
82	Fertilizer
83	Fertilizer
84	Fertilizer
85	Salicylic Acid
86	Salicylic Acid
87	Salicylic Acid
88	Salicylic Acid
89	Crushed Fine Willow Root
90	Crushed Fine Willow Root
91	Crushed Fine Willow Root
92	Crushed Fine Willow Root
93	Salicylic Acid and Fertilizer
94	Salicylic Acid and Fertilizer
95	Salicylic Acid and Fertilizer
96	Salicylic Acid and Fertilizer
97	Crushed Fine Willow Root and Fertilizer
98	Crushed Fine Willow Root and Fertilizer
99	Crushed Fine Willow Root and Fertilizer
100	Crushed Fine Willow Root and Fertilizer

Table 6: List of Fresh-Diesel Spiked Microcosms

20 °C Clean Kaltag Soil Spiked with Diesel

Microcosm Number	Amendments
25	Control
26	Control
27	Control
28	Control
29	Fertilizer
30	Fertilizer
31	Fertilizer
32	Fertilizer
33	Salicylic Acid
34	Salicylic Acid
35	Salicylic Acid
36	Salicylic Acid
37	Crushed Fine Willow Root
38	Crushed Fine Willow Root
39	Crushed Fine Willow Root
40	Crushed Fine Willow Root
41	Salicylic Acid and Fertilizer
42	Salicylic Acid and Fertilizer
43	Salicylic Acid and Fertilizer
44	Salicylic Acid and Fertilizer
45	Crushed Fine Willow Root and Fertilizer
46	Crushed Fine Willow Root and Fertilizer
47	Crushed Fine Willow Root and Fertilizer
48	Crushed Fine Willow Root and Fertilizer

4 °C Clean Kaltag Soil Spiked with Diesel

Microcosm Number	Amendments
101	Control
102	Control
103	Control
104	Control
105	Fertilizer
106	Fertilizer
107	Fertilizer
108	Fertilizer
109	Salicylic Acid
110	Salicylic Acid
111	Salicylic Acid
112	Salicylic Acid
113	Crushed Fine Willow Root
114	Crushed Fine Willow Root
115	Crushed Fine Willow Root
116	Crushed Fine Willow Root
117	Salicylic Acid and Fertilizer
118	Salicylic Acid and Fertilizer
119	Salicylic Acid and Fertilizer
120	Salicylic Acid and Fertilizer
121	Crushed Fine Willow Root and Fertilizer
122	Crushed Fine Willow Root and Fertilizer
123	Crushed Fine Willow Root and Fertilizer
124	Crushed Fine Willow Root and Fertilizer

Table 7: List of Clean Soil Microcosms

20 °C Clean Kaltag Soil

Microcosm Number	Amendments
49	Control
50	Control
51	Control
52	Fertilizer
53	Fertilizer
54	Fertilizer
55	Salicylic Acid
56	Salicylic Acid
57	Salicylic Acid
58	Crushed Fine Willow Root
59	Crushed Fine Willow Root
60	Salicylic Acid and Fertilizer
61	Salicylic Acid and Fertilizer
62	Crushed Fine Willow Root and Fertilizer
63	Crushed Fine Willow Root and Fertilizer
64	Crushed Fine Willow Root and Fertilizer

4 °C Clean Kaltag Soil

Microcosm Number	Amendments
125	Control
126	Control
127	Control
128	Fertilizer
129	Fertilizer
130	Fertilizer
131	Salicylic Acid
132	Salicylic Acid
133	Salicylic Acid
134	Crushed Fine Willow Root
135	Crushed Fine Willow Root
136	Salicylic Acid and Fertilizer
137	Salicylic Acid and Fertilizer
138	Crushed Fine Willow Root and Fertilizer
139	Crushed Fine Willow Root and Fertilizer
140	Crushed Fine Willow Root and Fertilizer

Table 8: List of Fresh-Diesel Spiked, Sterilized Soil Microcosms

20 °C Sterilized Clean Kaltag Soil Spiked with Diesel

Microcosm Number	Amendments
65	Control
66	Control
67	Fertilizer
68	Fertilizer
69	Salicylic Acid
70	Salicylic Acid
71	Crushed Fine Willow Root
72	Crushed Fine Willow Root
73	Salicylic Acid and Fertilizer
74	Salicylic Acid and Fertilizer
75	Crushed Fine Willow Root and Fertilizer
76	Crushed Fine Willow Root and Fertilizer

4 °C Sterilized Clean Kaltag Soil Spiked with Diesel

Microcosm Number	Amendments
141	Control
142	Control
143	Fertilizer
144	Fertilizer
145	Salicylic Acid
146	Salicylic Acid
147	Crushed Fine Willow Root
148	Crushed Fine Willow Root
149	Salicylic Acid and Fertilizer
150	Salicylic Acid and Fertilizer
151	Crushed Fine Willow Root and Fertilizer
152	Crushed Fine Willow Root and Fertilizer

Appendix B

Soil Moisture Results

As seen in Table 9, 10, 11 and 12, the moisture content was analyzed for all soils one month prior to the start of the experiment, on Day 1 of the experiment, and also on Day 45. Results showed that there were no significant decreases in moisture content, and in many cases the moisture content increased.

Table 9: Soil Moisture Content in Weathered-Diesel Microcosms

20 °C	**Prior to Start**	**Day 1**	**Day 45**
Control	34%	*	35%
Fertilizer	34%	30%	35%
Salicylic Acid	34%	31%	34%
Crushed Fine Willow Root	34%	31%	35%
Salicylic Acid and Fertilizer	34%	*	33%
Crushed Fine Willow Root and Fertilizer	34%	33%	34%
4 °C	**Prior to Start**	**Day 1**	**Day 45**
Control	34%	35%	34%
Fertilizer	34%	35%	36%
Salicylic Acid	34%	35%	34%
Crushed Fine Willow Root	34%	33%	33%
Salicylic Acid and Fertilizer	34%	35%	35%
Crushed Fine Willow Root and Fertilizer	34%	35%	33%

*No measurements available

Table 10: Soil Moisture Content in Fresh-Diesel Spiked Soil Microcosms

20 °C	**Prior to Start**	**Day 1**	**Day 45**
Control	44%	39%	46%
Fertilizer	44%	39%	42%
Salicylic Acid	44%	38%	43%
Crushed Fine Willow Root	44%	41%	44%
Salicylic Acid and Fertilizer	44%	43%	47%
Crushed Fine Willow Root and Fertilizer	44%	44%	47%
4 °C	**Prior to Start**	**Day 1**	**Day 45**
Control	44%	41%	47%
Fertilizer	44%	48%	48%
Salicylic Acid	44%	46%	46%
Crushed Fine Willow Root	44%	46%	47%
Salicylic Acid and Fertilizer	44%	45%	46%
Crushed Fine Willow Root and Fertilizer	44%	48%	45%

Table 11: Soil Moisture Content in Clean Soil Microcosms

20 °C	**Prior to Start**	**Day 1**	**Day 45**
Control	44%	42%	46%
Fertilizer	44%	44%	45%
Salicylic Acid	44%	42%	44%
Crushed Fine Willow Root	44%	45%	45%
Salicylic Acid and Fertilizer	44%	32%	45%
Crushed Fine Willow Root and Fertilizer	44%	44%	43%

Table 11 Continued

4 °C	Prior to Start	Day 1	Day 45
Control	44%	56%	43%
Fertilizer	44%	55%	40%
Salicylic Acid	44%	55%	41%
Crushed Fine Willow Root	44%	53%	45%
Salicylic Acid and Fertilizer	44%	53%	47%
Crushed Fine Willow Root and Fertilizer	44%	54%	46%

Table 12: Soil Moisture Content in Fresh Diesel-Spiked, Sterilized Microcosms

20 °C	Prior to Start	Day 1	Day 45
Control	44%	50%	44%
Fertilizer	44%	46%	43%
Salicylic Acid	44%	53%	52%
Crushed Fine Willow Root	44%	42%	55%
Salicylic Acid and Fertilizer	44%	43%	52%
Crushed Fine Willow Root and Fertilizer	44%	50%	55%
4 °C	**Prior to Start**	**Day 1**	**Day 45**
Control	44%	53%	43%
Fertilizer	44%	56%	45%
Salicylic Acid	44%	56%	42%
Crushed Fine Willow Root	44%	57%	42%
Salicylic Acid and Fertilizer	44%	53%	43%
Crushed Fine Willow Root and Fertilizer	44%	56%	45%

Appendix C

Multivariate Statistics

A multivariate approach is appropriate when there is more than one characteristic used to describe a sample and when simultaneously analyzing characteristics (McGarigal et al., 2000). The multivariate statistics offer several advantages for analyzing complex data sets, especially data associated with biological systems (McGarigal et al., 2000).

1) Able to accurately reflect the true multi-dimensional nature of biological systems
2) Able to deal with large data sets with multiple variables through summarizing the redundancy
3) Multivariate analysis provides rules allowing for the optimal combination of variables
4) Detects and quantifies multivariate patterns that result from the correlational structure of the variable set
5) Takes complex data sets and explores them for patterns and relationships from which hypotheses are generated and can be tested
6) Through post-hoc comparisons can explore the statistical significance of possible independent and dependent variable relationships
7) By controlling the experimentwise error rate (EER), the probability that one or more in a series of significant tests results in a Type I error, is able to provide a solution to multiple comparison problems

Multivariate statistical analysis is an assortment of descriptive and inferential statistics (Harris, 2001; McCarigal et al., 2000). Descriptive statistics provides the rules that allow for the optimal combination of variables (factors, principal components); the optimal way of combining

the variables depends on the specific technique (Harris, 2001). Inferential statistics provides solutions to multiple comparisons (Harris, 2001). As a result, the multivariate statistical approach will search through data containing multiple variables looking for patterns and relationships from which hypotheses can be generated and tested (McGarigal et al., 2000; Rencher, 2002). When searching through the data, multivariate analysis will assess variables for linear combinations for a main effect (Harris, 2001). A benefit of this approach is that it can prevent the individual employing the technique from reading too much into the data (Rencher, 2002).

The ultimate goal of multivariate statistics is to simplify data sets, relying on an empirical approach, so that important parameters can be isolated from noise that exists in the data (Dempster, 1971; Rencher 2002). The data set for a multivariate analysis will consist of dependent variables that are to be related to independent and dependent variables (Dempster, 1971). This also helps meet the requirement of having a flexible group of hypothetical distributions of free variables indexed by fixed variables (Dempster, 1971). It will be critical to know the form in which the value of the variable exists, which means that the variable will need to be defined as dichotomous (example yes or no), ordered polytomy (example good, fair, poor), non-ordered polytomy (example different chemical treatments), or as a numerical response (Dempster, 1971).

The data set will have a logical structure, which is important to understand as it will allow for better interpretation of substantive information, as a result of the interrelationships that exists among the variables (Dempster, 1971). Each data set will be associated with *a priori* knowledge (Dempster, 1971; Goodman et al., 2014):

1) Distinction between free variables (in response to controlled conditions) and fixed variables (deliberately set)
2) Conditions of symmetry which allows for a combination system describing multiple situations

Once variables are found to be statistically significant (from post hoc comparison) it will then be known which combination of variables from the data set resulted in the greatest evidence against the null hypothesis, and by using the *a priori* knowledge we can compare the efficacy (Harris, 2001).

Data cleansing from the data set is acceptable, which is the removing of outlying values or those known to be impossible to have occurred. A typical approach for data cleaning is to review a sample distribution and identify outliers or impossible variables (Dempster, 1971).

Appendix D

Partial Least Squares

PLS is a soft modeling approach; a soft model applies when theoretical knowledge is limited and distributional assumptions are not applicable (Lohmöller, 1989). Soft models include observed (manifest) variables (DRO loss) and latent variables (temperature and soil amendments) which are related through linear equations (Lohmöller, 1989). PLS methods use latent variables (underlying factors) to model the relations between observed variables (Rosipal and Krämer, 2006; Tobias, 1997). The underlying assumption to all PLS methods is that observed data are the result of a process driven by latent variables (Rosipal and Krämer, 2006). In general, PLS extracts the latent factors which can account for the greatest variation in the observed variable while being able to model the responses (Tobias, 1997).

The PLS approach can be considered an extension of multiple linear regression modeling (Tobias, 1997). It uses a limited information approach, as such it assumes nothing about the scale or population of measurements and works without assumptions of distribution (Haenlein and Kaplan, 2004). The PLS method has the benefit of being used for theory confirmation, determining if relationships exist and suggesting items for later testing (Chin, 1998).

Results of PLS modeling can be displayed graphically, there is a multitude of ways to present the data. To determine the correct number of factors (PCs) required to explain a PLS model, two graphs are needed, a variance graph and loadings plot (Westad et al., 2002). Factors are vectors that are variance scaled in a variable space. Factors are formed through the projection of objects onto axes that have particular features (Esbensen et al., 2002). Figure 7 shows how many factors are required to explain variance in the data, this data may also be presented in table format.

Figure 9 shows how much of the variance is explained with each additional factor; based on the figure it appears that 2 factors are sufficient to explain the variance. When little change is seen in the amount of explained variance, the graph is indicating the modeling of noise (Westad et al., 2002). The calibration or calibrated explained variance described in Figure 9, and throughout other figures, is the result of fitting the model to the available data and observing how well the model describes the data (CAMO, 2016). Validation or validated explained variance determines how well the model used can operate for future samples that are from the same population as the calibrated samples (CAMO, 2016). The closer validation results are to the calibrated results, the more reliable the model.

For each factor the referenced (collected data) and predicted values can be placed on a simple scatter plot (e.g. Figure 10). This allows for a determination of PLS's ability to model the data. It also is a way to visually find outliers. The correlation between the referenced and predicted variables is determined by dividing the covariance (measure of linear association between two variables, e.g. the observed and predicted percent DRO loss) by the product of their respective standard deviations (Esbensen et al., 2002). The scatter graph can be assessed through evaluation of the R-squares (R^2) for each individual dependent variable (Chin, 1998). The R-squares can also be used to determine the influence of various latent variables on the dependent variable (Chin, 1998). An R-square value ranges between 0 (0%) and 1 (100%), the closer the R-square value is to one the better the fit of the model and its ability to explain total variation.

Correlation can be represented through correlation loadings figures (e.g. Figure 11), showing the correlation between two independent variables (principal components or factors) and the dependent variable (Mevik and Wehrens, 2007). The graph is essentially a scatter plot of two

score sets with each point corresponding to an X variable (Mevik and Wehrens, 2007). Factor 1 (PC 1) lies along the x-axis while Factor 2 (PC 2) is represented by the y-axis.

Factor 1 and Factor 2 are independent variables. Variables that exist close to a factor axis show the variable is significant only to that factor (Esbensen et al., 2002). Adjacent to each factor heading along the x and y-axis, the percentage of explained variance is shown (i.e. Factor-1 (96%, 55%)). The first percentage is the factor's ability to explain the independent variable and the second listed percentage is the ability of the factor to explain the dependent variable (%DRO Loss). The ability of both factors together to describe the dependent variable can be determined by adding each of the percentages, for example together Factor 1 and Factor 2 describe 84% of the percent of DRO loss in Figure 11. Factors cannot exceed 100% explained variance of the x-axis and the dependent variable.

There are two concentric ovals which correspond to the explained variance or how much variance the model took into account; the inner oval shows 50% explained variance of the dependent variable while the outer is 100% explained variance of the dependent variable (Mevik and Wehrens, 2007). Points located near the origin are considered to have little importance (such as fertilizer in Figure 11) while those further out, such as temperature and willow roots are of greater importance (Esbensen et al, 2002). Independent variables that exist on the same side of the origin are positively correlated to each other, while those on the opposite side show negative correlation (Esbensen et al., 2002). As an example, in Figure 11, the presence of crushed fine willow root positively correlated with loss in DRO while salicylic acid (which is on the opposite side of the origin) negatively correlated with DRO loss.

Appendix E

Example of Unscrambler® Matrix

Table 13: Example of Unscrambler® Matrix

Parameter	Soil Type	Temp. (°C)	Fert.	SA	WR	Average DRO (mg/kg)	Actual DRO (mg/kg)	% DRO Loss
Control	Weathered	20	-1	-1	-1	55000	43997	42
Control	Weathered	20	-1	-1	-1	55000	65112	61
Fert.	Weathered	20	1	-1	-1	55000	50947	
Fert.	Weathered	20	1	-1	-1	55000	51284	57
SA	Weathered	20	-1	1	-1	55000	47503	44
SA	Weathered	20	-1	1	-1	55000	41283	36
WR	Weathered	20	-1	-1	1	55000	52342	56
WR	Weathered	20	-1	-1	1	55000	49031	67
SA+Fert.	Weathered	20	1	1	-1	55000	43970	45
SA+Fert.	Weathered	20	1	1	-1	55000	48909	27
WR+Fert.	Weathered	20	1	-1	1	55000	61237	85
WR+Fert.	Weathered	20	1	-1	1	55000	61104	68
Control	Weathered	4	-1	-1	-1	55000	50506	24
Control	Weathered	4	-1	-1	-1	55000	48479	13
Fert.	Weathered	4	1	-1	-1	55000	45839	31
Fert.	Weathered	4	1	-1	-1	55000	72990	37
SA	Weathered	4	-1	1	-1	55000	48924	14
SA	Weathered	4	-1	1	-1	55000	42431	14
WR	Weathered	4	-1	-1	1	55000	48991	16
WR	Weathered	4	-1	-1	1	55000	57328	23
SA+Fert.	Weathered	4	1	1	-1	55000	60734	11
SA+Fert.	Weathered	4	1	1	-1	55000	69837	-1
WR+Fert.	Weathered	4	1	-1	1	55000	79427	46
WR+Fert.	Weathered	4	1	-1	1	55000	77235	40

Appendix F

Supplemental GC/MS Results

Table 14: DRO Concentrations in Weathered-Diesel Microcosms

20°C Microcosm	Parameter	Day 1 (mg/kg)	Day 90 (mg/kg)	Percent DRO Loss
3	Control	43997	25600	42%
4	Control	65112	25260	61%
7	Fert.	50947	Missing	Missing
8	Fert.	51284	21808	57%
11	SA	47503	26650	44%
12	SA	41283	26369	36%
15	WR	52342	22789	56%
16	WR	49031	16202	67%
19	SA&Fert.	43970	24004	45%
20	SA&Fert.	48909	35481	27%
23	WR&Fert.	61237	9261	85%
24	WR&Fert.	61104	19412	68%
4°C Microcosm	**Parameter**	**Day 1 (mg/kg)**	**Day 90 (mg/kg)**	**Percent DRO Loss**
79	Control	50506	38404	24%
80	Control	48479	42197	13%
83	Fert.	45839	31464	31%
84	Fert.	72990	46340	36%
87	SA	48924	41892	12%
88	SA	42431	36490	14%
91	WR	48991	41053	16%
92	WR	57328	44198	23%
95	SA&Fert.	60734	54079	11%
96	SA&Fert.	69837	70376	-1%
23	WR&Fert.	79427	42943	46%
24	WR&Fert.	77235	46090	40%

Table 15: DRO Concentrations in Fresh-Diesel Spiked Microcosms

20 °C Microcosm	Parameter	Day 1 (mg/kg)	Day 90 (mg/kg)	Percent DRO Loss
27	Control	384	47	88%
28	Control	1249	155	88%
31	Fert.	1454	151	90%
32	Fert.	836	153	82%
35	SA	783	159	77%
36	SA	1002	126	87%
39	WR	1474	155	89%
40	WR	650	250	61%
43	SA&Fert.	1306	279	79%
44	SA&Fert.	1395	190	86%
47	WR&Fert.	643	183	71%
48	WR&Fert.	1526	186	88%
4 °C Microcosm	**Parameter**	**Day 1 (mg/kg)**	**Day 90 (mg/kg)**	**Percent DRO Loss**
103	Control	1527	67	96%
104	Control	2118	287	86%
107	Fert.	2497	0	100%
108	Fert.	2709	211	92%
111	SA	2609	2159	17%
112	SA	2367	244	90%
115	WR	2805	192	93%
116	WR	1598	235	85%
119	SA&Fert.	2492	275	89%
120	SA&Fert.	838	258	69%
123	WR&Fert.	1835	210	89%
124	WR&Fert.	1798	108	94%

Table 16: Average DRO Loss from Weathered-Diesel Microcosms Over 90 Days

	20 °C Percent DRO Loss	4 °C Percent DRO Loss
Control	53.4%	18.6%
Fertilizer	57.3%	34.5%
Salicylic Acid	40.3%	14.2%
Willow Root	61.5%	19.8%
Salicylic Acid + Fertilizer	36.0%	4.7%
Willow Root + Fertilizer	76.6%	43.2%

GC/MS analysis was performed for all soil types, except for clean soil microcosms. The results from the fresh diesel-spiked microcosms with or without sterilization could not be modeled using PLS regression modeling; there were no valid results. The reason for this is most likely volatilization of the diesel, which already started when mixing diesel with the soils. High removal rates were observed for almost all microcosms, with little effect of soil amendments, as shown in Figure 36 and Figure 37, though for salicylic acid at 4 ℃, lower diesel removal was observed. Results for 4 ℃ fresh diesel-spiked, sterilized soil for the control and fertilizer amended microcosms are missing due to accidental sample destruction.

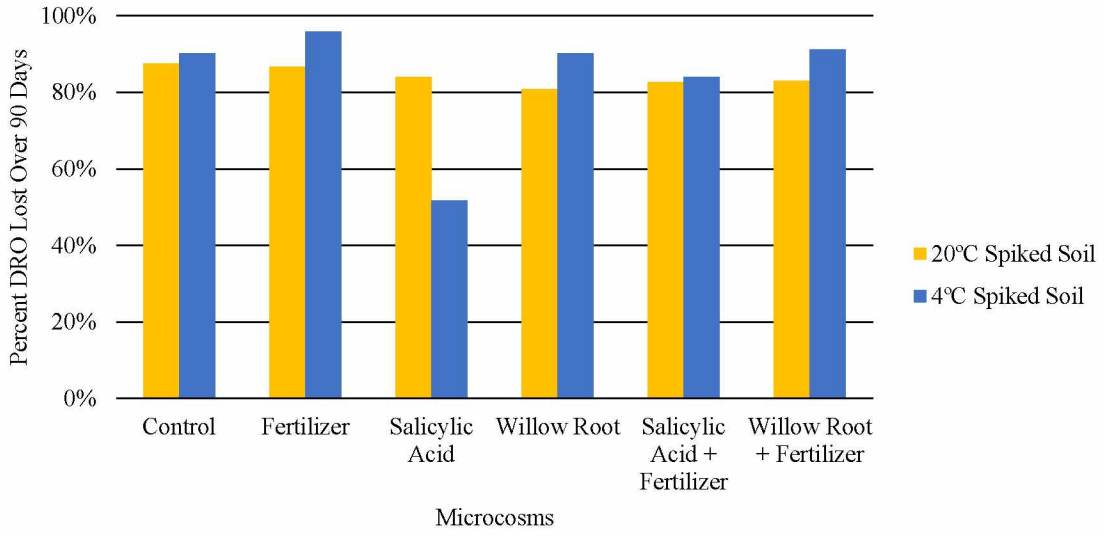

Figure 36: Total DRO Loss from Fresh-Diesel Spiked Microcosms Over 90 Days

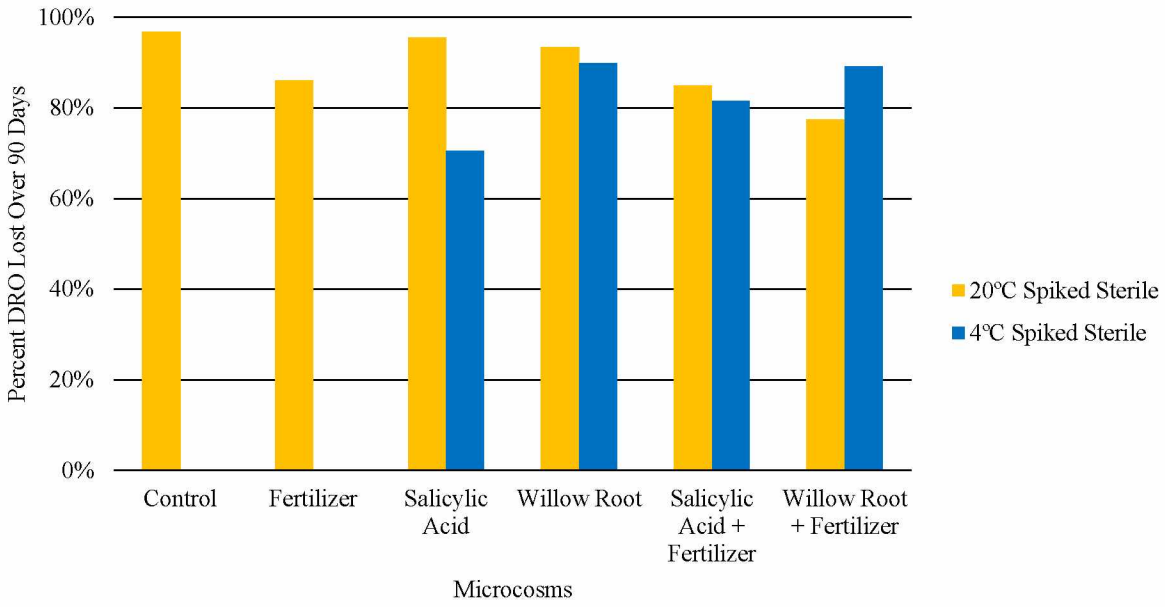

Figure 37: Total DRO Loss from Fresh-Diesel Spiked, Sterile Microcosms Over 90 Days

Appendix G

Supplemental MPN Results

Microcosms with fresh diesel-spiked, sterilized soil did not remain sterile under both temperature conditions as shown by Figure 38 and Figure 39. While no DDM were detected on day 3, DDM numbers on later days were of comparable magnitude as in microcosms without sterilization.

At 20 ºC crushed fine willow root with or without fertilizer had the highest MPN; and lowest MPN was seen with salicylic acid and fertilizer. At 4 ºC, DDM were measured at lower numbers than at 20 ºC. For salicylic acid and fertilizer amended microcosms, DDM were detected at Day 48 but not at Day 89.

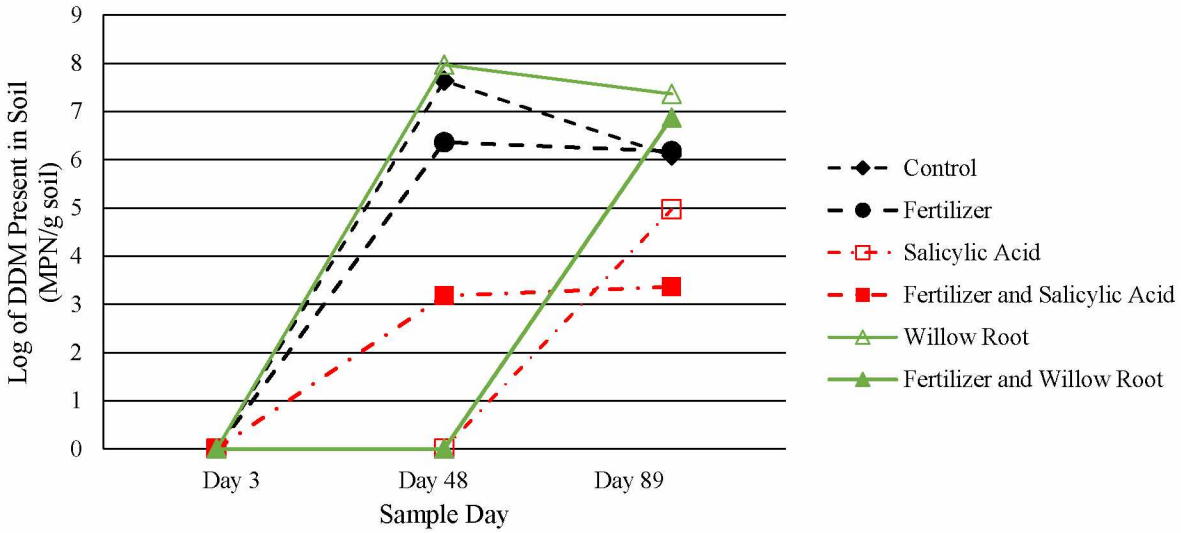

Figure 38: Concentration of DDM in Fresh-Diesel Spiked, Sterile Microcosms at 20 ºC

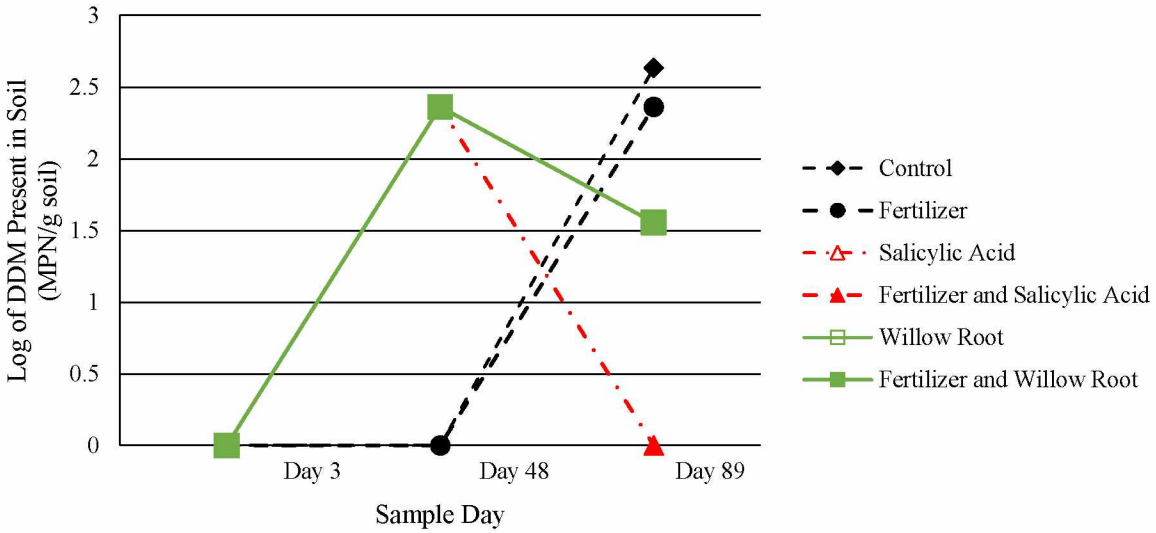

Figure 39: Concentration of DDM in Fresh-Diesel Spiked, Sterile Microcosms at 4 ºC

PLS regression was unable to accurately model MPN data for all soil types. Attempts were made to model all of the MPN data (Figure 40), as well as to model individual soil sets (Figure 41), but all failed; as such, definitive conclusions cannot be made regarding the MPN data.

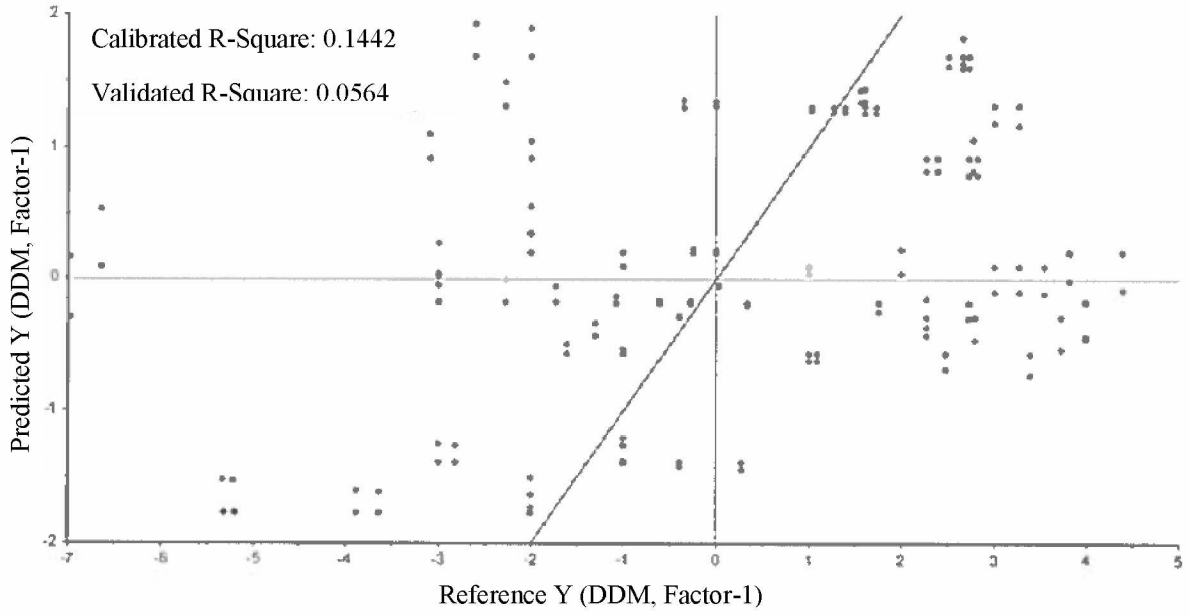

Figure 40: PLS Regression Modeling of DDM concentration in all Microcosms

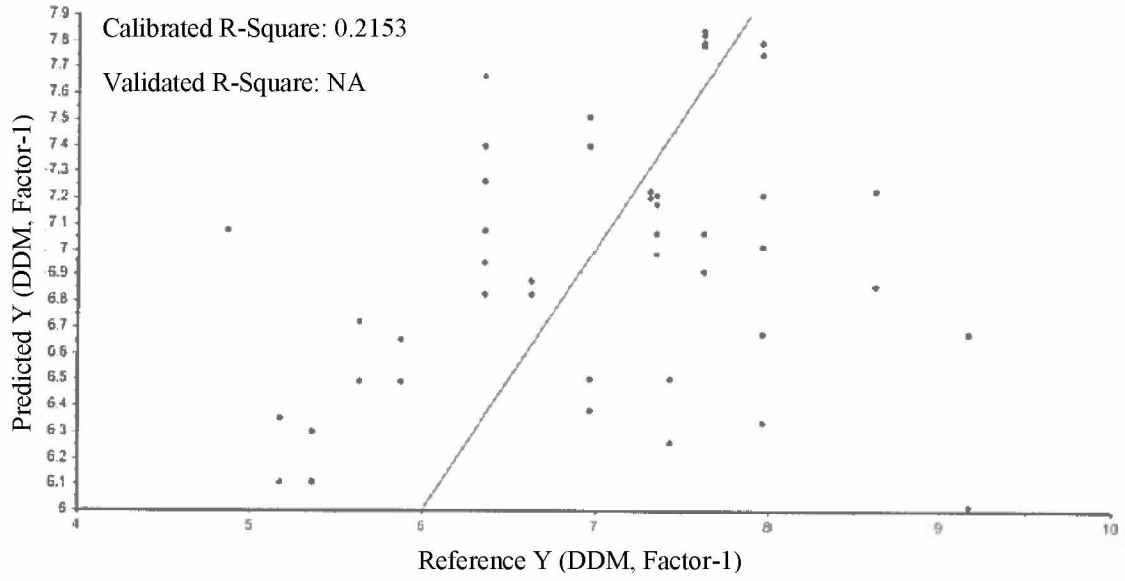

Figure 41: PLS Regression Modeling of DDM concentration in Weathered-Diesel Microcosms

Appendix H

Supporting LI-COR Results

Clean soil incubated at 20 ºC (Figure 42) amended with salicylic acid plus fertilizer had the highest respiration peak of all samples at Day 9 with 1,000 g CO_2. At 4 ºC (Figure 43), salicylic acid (415 g CO_2) and salicylic acid + fertilizer (356 g CO_2) resulted in a respiration peak at Day 41. Baseline respiration rates were similar at both 20 ºC and 4 ºC for clean soil microcosms.

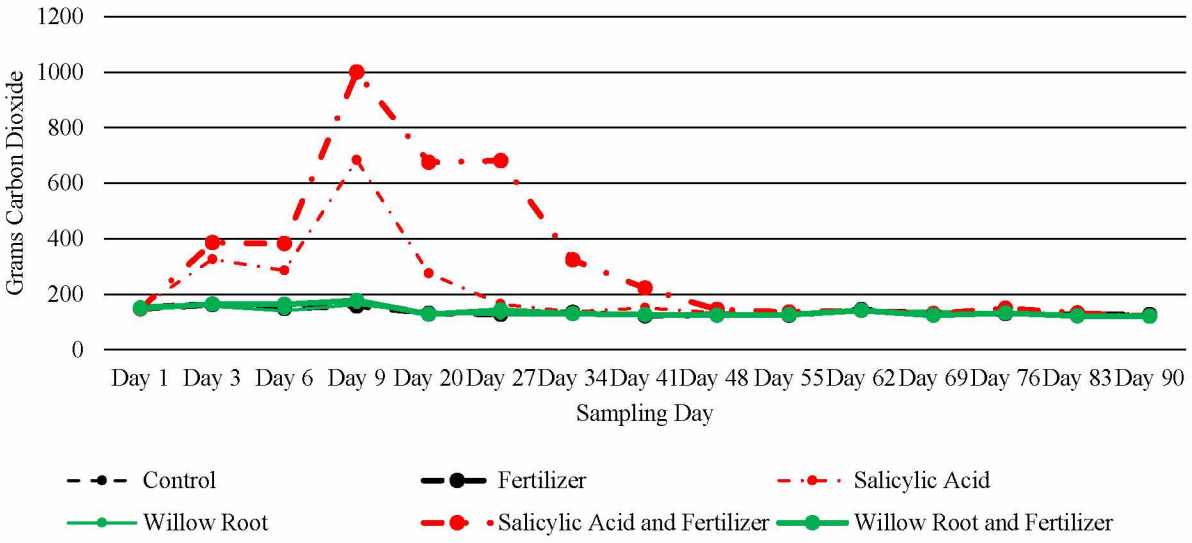

Figure 42: Daily CO_2 Production in Clean Soil Microcosms at 20 ºC

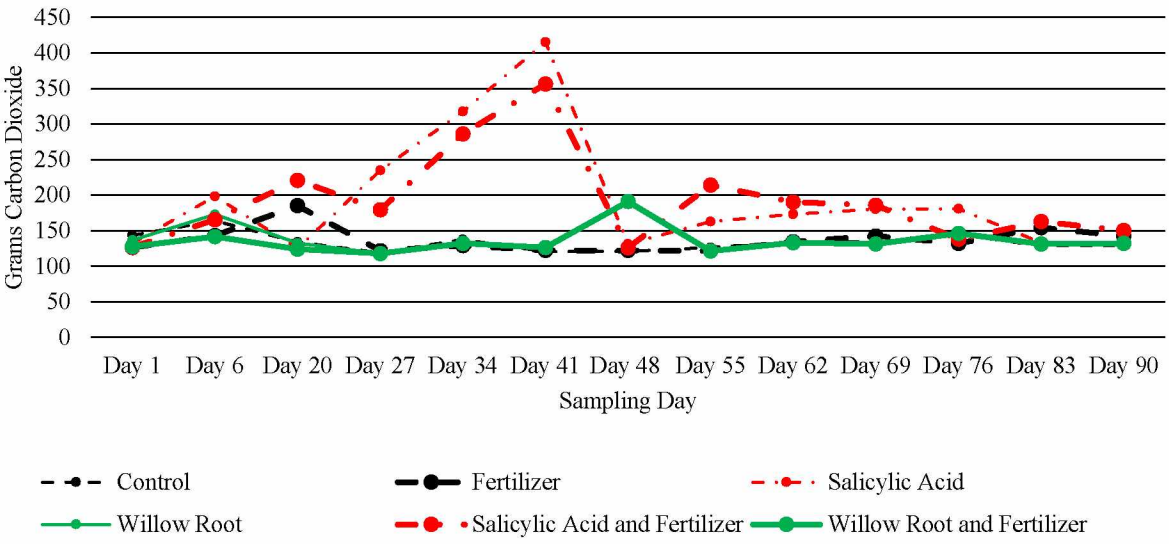

Figure 43: Daily CO_2 Production in Clean Soil Microcosms at 4 °C

The PLS regression model for clean soil respiration required that microcosm # 61 (20 °C salicylic acid and fertilizer) be removed as it was an extreme outlier. It was determined that this sample point was an outlier as it was at a greater distance away from other samples points in the regression modeling (Figure 44), including point 56. Figure 45 shows the PLS model of the data; the model was very effective with collected data having an R^2 values of 0.9732 and 0.9089 for calibration and validation respectively. Two factors were found be optimal for describing the variance in the data. Temperature (Factor 1) (Figure 45) is capable of describing 96% of the variance in the x variable and 10% of the response variable. Soil amendments (Factor 2) (Figure 45) only described 2% of the variable but described 75% of the response variable. The strongest influence on respiration came from the presence of salicylic acid. Overall, the two factors describe 98% of the x variable variance and 85% of the response variable.

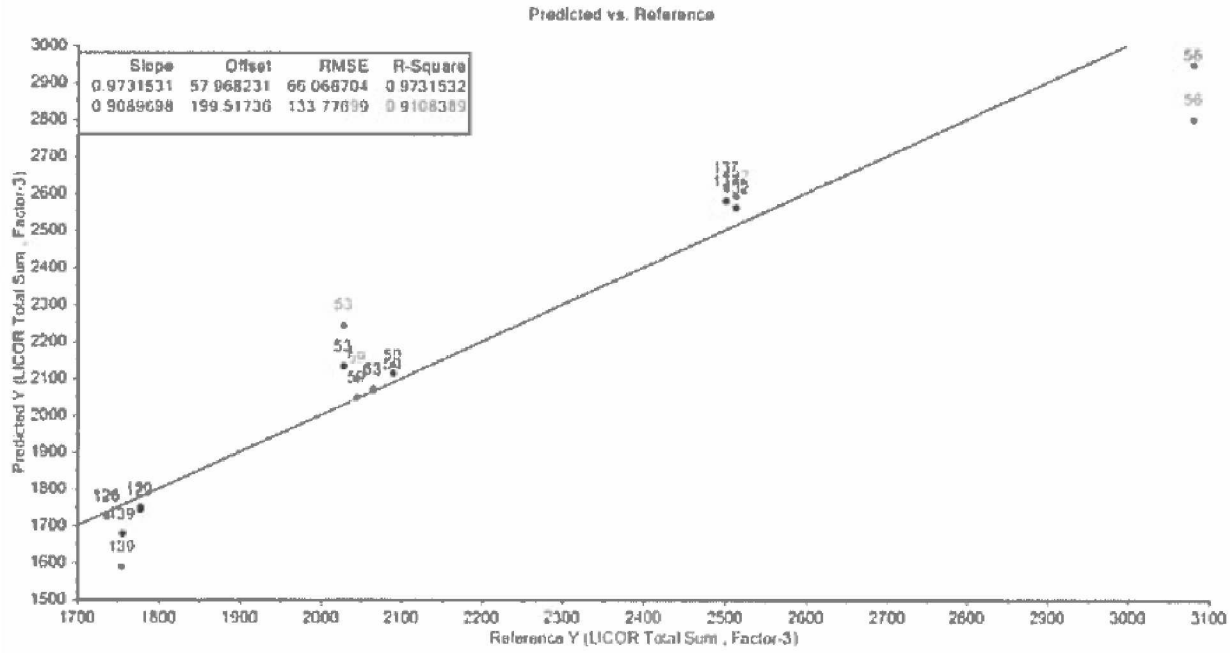

Figure 44: PLS Regression Modeling of CO_2 Results in Clean Soil Microcosms

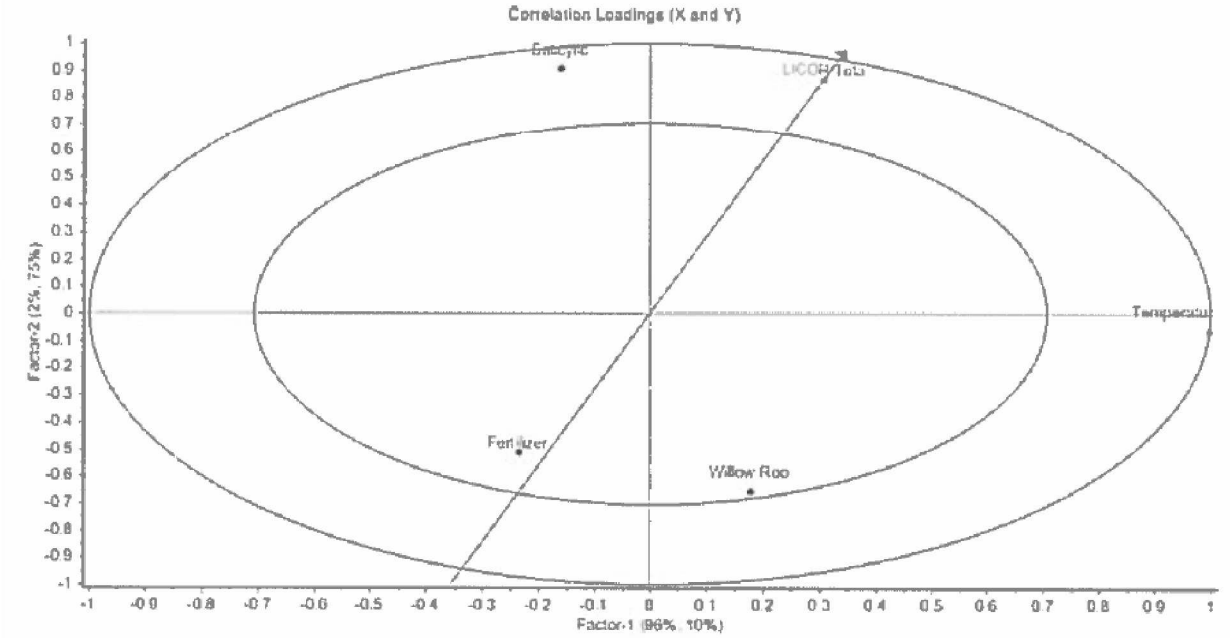

Figure 45: PLS Correlation Loadings for CO_2 Production in Clean Soil Microcosms